Impact of viral immunomodulatory proteins

At the level of the cell and the whole animal

V. L. de Oliveira

Dissertation presented to obtain the Ph.D degree in Biology

by Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
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Knowledge Creation

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" Life is like riding a bicycle.
To keep your balance, you must keep moving"

Albert Einstein

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Contents

1 Resumo

1.1 Impacto de proteínas virais imunomoduladoras ao nível da célula e do animal inteiro

Cerca de 50% do genoma dos vírus de DNA evoluiu direcionado para a manipulação de importantes funções celulares do hospedeiro. Estas estratégias são muito diversas e conferem ao vírus vantagens importantes sobre o sistema imunitário do hospedeiro. Esses genes são, por isso, potenciais fontes de informação para a geração de novos fármacos dirigidos à manipulação da resposta imunológica na saúde e na doença. Esta tese centra-se na análise da função de dois genes virais distintos, ambos com funções imunomoduladoras. O gene do Vírus da Peste Suína Africana codificado pela "open reading frame" I329L (ORF I329L), e o gene do vírus herpes-gama-68 de murino codificado pela "open reading frame" M2 (ORF M2). Ambos os vírus são conhecidos por codificar várias proteínas capazes de manipular componentes vitais da resposta antiviral. Neste trabalho nós demonstramos que tanto a ORF I329L quanto ORF M2 são capazes de manipular a imunidade inata ou adquirida.

O vírus da peste suína africana (ASFV) é o único membro da nova família Asfarviridae. Pode dizer-se que este vírus atingiu um equilíbrio com o hospedeiro na qual causa sintomas mínimos de doença sendo no entanto capaz de evitar sua eliminação através da manipulação do sistema imunológico.

Com tropismo para os macrófagos, o vírus causa uma infeção persistente e assintomática nos seus hospedeiros naturais – potamoqueros e javalis selvagens. Em contraste, a infecção de porcos domésticos com o vírus da peste suína africana resulta numa doença hemorrágica fatal com apoptose linfoide massiva. A peste suína africana é, por isso, considerada como uma "doença veterinária emergente".

Além de infetar suínos, o vírus da Peste Suína Africana infecta também a carraça, o seu vector de transmissão invertebrado. A replicação do vírus da Peste Suína Africana em organismos vertebrados e invertebrados sugere que o vírus terá desenvolvido estratégias de evasão imunológica para ambos os hospedeiros.

Para além disso, o facto de que em hospedeiros vertebrados o vírus da peste suína africana infectar unicamente uma célula tão importante na resposta imunitária inata, os macrófagos, sugere a presença de genes antagonistas da sinalização via TLR no genoma do ASFV.

A primeira parte desta tese descreve a procura por um gene capaz de manipular um importante componente da resposta controlada pelos receptores "Toll" e "Toll-like" no genoma do vírus da peste suína africana. Para tal, foi efetuada uma procura bioinformática por um gene/proteína com homologia com algum interveniente das vias acima citadas. Esta procura resultou na identificação da ORF I329L, uma glicoproteína

hipotética com um domínio transmembranar simples. Esta hipótese foi confirmada por ensaios bioquímicos e de biologia celular.
Usando um sistema de gene repórter foi possível demonstrar que a proteína codificada pela ORF I329L inibe a ativação do NFkB e IFN-β desencadeada pela estimulação do TLR3. Este mesmo sistema revelou que o mecanismo de inibição é independente de MyD88 e que é dirigido para a manipulação do intermediário de sinalização TRIF.
Alvos celulares adicionais não foram excluídos já que muitos genes de evasão virais são multifuncionais. Finalmente, a expressão do gene I329L em células de mamífero resulta na inibição da expressão de RANTES após estimulação do TLR3 com um análogo do RNA de cadeia dupla, Poly I:C. Em suma, foi identificado e caracterizado um novo gene de evasão viral do Vírus da Peste Suína Africana, a ORF I329L, com a função de inibir a resposta desencadeada pelo receptor TLR3.
O vírus herpes-gama-68 de murino (MHV-68) é um membro do omnipresente e bem-sucedido grupo de vírus herpes. O MHV-68 é muito semelhante as outros vírus que causam infecções em humanos, como o vírus Epstein-Barr (EBV) e o vírus associado ao sarcoma de Kaposi (KSHV), produzindo um tipo de doença conhecida como mononucleose infeciosa aguda. Em ratinhos este vírus persiste latente em linfócitos B.
Muitos dos estudos sobre a resposta imunológica a infeções virais com vírus herpes-gama-68 de murino, provém da análise da patogénese viral em diferentes ratinhos geneticamente modificados. Estes são, na sua maioria, desprovidos de um ou de vários componentes do sistema imunológico adaptativo.

A construção de ratinhos transgénicos é uma estratégia poderosa para estudar os mecanismos de imunidade *in vivo*. O uso de promotores específicos que restringem a expressão do gene de interesse a determinado tipo de células é um refinamento que ajuda a superar os efeitos indesejáveis de uma transgenesis global, presente em todas as células do organismo.
Assim, a expressão restrita da ORF M2 em linfócitos B constitui uma nova abordagem *in vivo* para estudar o mecanismo de manipulação das defesas do hospedeiro por este gene.
Esta abordagem tem a vantagem de possibilitar estudos em contexto de infeção e constitui um novo sistema para o estudo do impacto de uma única proteína viral *in vivo*, com expressão restrita durante as fases aguda e latente do ciclo de infeção viral.
O gene M2 do vírus MHV-68 está envolvido no estabelecimento, manutenção e reativação da latência em células B. Mais ainda, está descrito que a proteína M2 interage com o sistema de sinalização VAV, o que sugere a manipulação dos receptores de células B através desta via para regular a ativação, proliferação e, ou sobrevivência das células B infetadas. Após analisar os marcadores de superfície dos linfócitos B de ratinhos transgénicos não se verificaram diferenças significativas nas subpopulações de células B da medula óssea ou do baço sugerindo

assim que a ORF M2 não tem qualquer impacto ndesenvolvimento destas células. Por sua vez, ratinhos transgénicos imunizados com o antigénio T-dependente (DNP-KLH) obtiveram níveis elevados, e estatisticamente significativos, de anticorpos IgM e IgG2a quando comparados com animais não modificados. Em particular, é importante salientar que, após a imunização com hemácias de carneiro, foi observado um decréscimo significativo da taxa de apoptose em linfócitos B. Estas observações sugerem que a ORF M2 possa ter funções ao nível do ciclo celular, em particular no aumento da sobrevivência das células.

A resposta do ratinho transgénico M2 à infeção com o vírus MHV-68 foi também explorada neste modelo. Para isso, ratinhos transgénicos e controles foram infectados com vírus não modificados ou com vírus mutantes, em que a ORF M2 foi eliminada. De seguida o número de células B com MHV-68 em latência no baço foi quantificado através da contagem de placas virais geradas a partir da reactivação do vírus presente em células B isoladas deste órgão. Observou-se assim um aumento da produção de vírus em ratinhos não transgénicos infectados com o vírus mutante deficiente em M2 numa fase final da infeção mas não numa fase inicial.

Este fenótipo não foi revertido em ratinhos M2 transgénicos infectados com vírus mutante ou infectados com vírus não modificado. Nestes últimos, a observação de altos títulos de reativação numa fase tardia da latência é consistente com a já demonstrada importância da proteína M2 como um immunodeterminante da imunidade celular protetora, assim como o seu impacto no estabelecimento da persistência.

Finalmente, uma considerável redução do número de células B em apoptose foi observada em ratinhos não transgénicos infectados com vírus MHV-68. Isto está de acordo com o que foi observado anteriormente com ratinhos transgénicos imunizados com hemácias de carneiro bem como com dados *in vitro* utilizando uma linha celular linfócitos B imaturos, WEHI-231 (Madureira *et al.,* 2005).

Com base nos resultados obtidos com o nosso modelo, sugerimos que M2 favorece a reativação e consequente transmissão do vírus ao aumentar a sensibilidade das células B infectadas em latência para a ativação induzida pelo recetor de imunoglobulinas (BCR) através da interação com VAV. Além disso, estes dados indicam que a ORF M2 pode funcionar como promotor da sobrevivência de células B ativadas.

2 Abstract

2.1 Impact of viral immunomodulatory proteins at the level of the cell and the whole animal

Up to fifty percentage of the genome of large DNA viruses have evolved to manipulate the function of important cell biological and immunological responses and so give the virus an advantage over the host cell. Such genes are potential sources of novel health care pharmaceuticals for manipulation of immune responses in health and diseases. This thesis focuses in exploring the function of two different viral immune modulators, the African Swine Fever Virus (AFSV) ORF I329L and the Murine Gamma herpes Virus (MHV-68), ORF M2 genes. Here, We demonstrate that ORF I329L and ORF M2 manipulate host innate and adaptive immunity, respectively.

The ASFV is the only member of a new virus family, the *Asfarviridae* with a tropism for macrophages and which in the wild life hosts the Bushpig and Warthog, and it causes an unapparent persistent infection. In contrast, infection of domestic pigs with ASFV results in a fatal haemorrhagic disease with massive lymphoid apoptosis, and so may be regarded as a "recently emerged veterinary disease". Importantly, the ASFV also infects an invertebrate vector, the soft-tick. The survival of ASFV in both vertebrates and invertebrates suggests that this virus has evolved "immune evasion" strategies for both of these hosts. This, and the fact that in the vertebrate host, the virus uniquely infects the central cell of the innate immune response, the macrophage, suggests the possibility of TLR signaling antagonists in the ASFV genome.

Thus, the first part of the thesis presents a successful search for an ASFV host evasion gene that inhibits an important component of the innate immune response controlled by the Toll-Like Receptors (TLRs). The work commenced with a bioinformatics search of the ASFV genome and, with only marginal sequence homology by basic informatic enquiry yielded ORF I329L as a candidate. The ORF I329L is predicted to be transmembrane glycoprotein, and this was confirmed by biochemical and cell biological investigations. Using luciferase reporter assays, I329L was then demonstrated to inhibit TLR3 stimulated activation of NFκB and IFN-β by a MyD88 independent mechanism consistent with the targeting of the intracellular signaling intermediate TRIF. Additional cellular targets of the gene other than TRIF are not ruled out, as many virus host evasion genes are multifunctional. Finally, and significantly, introduction of the gene into the cells resulted in an inhibition of RANTES secretion in response to stimulation with the viral dsRNA analogue poly I:C. In conclusion, ASFV ORF I329L is a novel virus host evasion gene evolved to diminish the host cell responses, controlled by TLR3.

The MHV-68 virus is a member of the ubiquitous and successful herpes virus

group and establishes persistence in murine B-lymphocytes. The MHV-68 behaves similarly to EBV and KSHV infections in humans, producing an acute mononucleosis-like illness and a pool of latently infected B cells. Much of what has been learned about the immune response to gamma-herpes virus infection with MHV-68 has come from evaluating its pathogenesis in different genetically modified mice lacking various components of the adaptive immune system. The construction of transgenic mice, on the other hand, is a powerful strategy to study mechanisms of immunity *in vivo*, and the use of specific promoters to restrict cellular expression to a certain cell type is an additional refinement, which may overcome the undesirable effects of whole body transgenesis. Thus, the B-cell restricted expression of the MHV-68 ORF M2 in the transgenic mice provides a novel approach to explore the mechanism and possible exploitation of this gene. Also it has the merit of being relevant in the context of an MHV-68 infection, providing a system to study the impact *in vivo* of a single virus protein with cell restricted transgenic expression during acute and latent phases of virus infection.

The M2 gene of MHV-68 was selected for B-cell restricted transgenic expression as it is thought to be involved in the establishment, maintenance and reactivation of latency in B cells. Moreover, M2 interacts with Vav signalling system, suggesting that M2 may modulate B-cell receptor-mediated signalling events through Vav to manipulate the activation, proliferation and/or survival of B cells. Analysing surface marker expression of these M2-transgenic mice, it was observed that the M2 transgene was without impact on the development of B cells, as there were no differences in B-cell subsets in the bone marrow or in the spleen. Transgenic mice immunized with the T dependent antigen (DNP-KLH) have statistically higher levels of both IgM and IgG2a antibodies than normal mice. Particularly significant was the observation that upon immunization with sheep red blood cells, there was a significant decrease in B cell apoptosis in the M2-transgenic.

The response of M2 transgenic mice to infection with the MHV-68 was also explored. Transgenic and wild-type mice were infected with wild-type and M2 deletion mutant viruses, and then the numbers of reactivation-competent virus plaques in the spleen were measured as an indication of the number of latently infected B cells. The yield of virus recovered in normal mice infected with the M2 deficient virus increased at the late, but not at earlier phase of infection. This feature was not observed in the M2 transgenic mouse, where the response to wild-type and M2 knockout viruses was similar. The observation of higher reactivation centres in the late phase of latency in transgenic mice infected with wild-type viruses is consistent with the proven importance of M2 as a determinant of protective cellular immunity, and thus with an impact on the establishment of persistence.

Finally, considerably less B cell apoptosis was observed in MHV-68 infected M2 transgenic mice than in MHV-68 infected normal mice, thereby

confirming our observation on sheep red blood cell immunized mice and the previous work of Madureira *et al.* in M2 transfected WEHI-231 B cell lines. Based on these results we suggest that M2, through its interaction with Vav, increases the sensitivity of the latently infected B cells to activation via the immunoglobulin receptor (BCR), and thus might favour reactivation and consequent virus transmission. In addition, these experiments suggest that M2 might function to promote the survival of activated B cells.

01 | General Introduction

1 Viruses & host's immune system

1.1 Viruses

"Marginalized", viruses do not belong to the five kingdoms of life; they are much smaller and much less complex than cells; they usually show up with little baggage, often wearing just a thin protein coat wrapped around a small cluster of genes ("bad news wrapped in protein", according to Peter Medawar). They do not exhibit autopoiesis, as they do not have the self-maintenance metabolic reactions of living systems. They live as obligate parasites and are only able to reproduce within living cells. However despite their apparent molecular simplicity, viruses have evolved sophisticated strategies to survive and propagate within their hosts. Viruses act like hijackers invading the host cell and taking over it in order to synthesize their proteins and replicate. When viruses infect, we must rely on our immune system, one of the most complex and remarkable achievements in the evolution of living systems.

1.2 Viral infection control

Microorganisms exist in a highly dynamic state of equilibrium (homeostasis) with their hosts, in which both immune and non-immune pathways contribute to control. Pathogen survival, in virus infections results in viral clearance and/or asymptomatic homeostasis. In this light, an infection can be viewed as a highly complex interaction that takes place between two partners: the host and the pathogen. The host vs. pathogen interaction has provided the driving force for the reciprocal evolution of the immune system and the many pathogen strategies for host evasion.

In the immune-competent individual, primary control of an acute infection is provided by both innate and adaptive immune responses **(Figure 1).** On the other hand, the immune system microenvironment is not completely sterilized and in many cases pathogens have adapted by using general and specific strategies to counteract the host immune attack. Thus the mammalian immune system has evolved several mechanisms to destroy viruses and virus-infected cells and, at the same time, stimulated the evolution of virus' countermeasures to neutralize or evade this host attack.

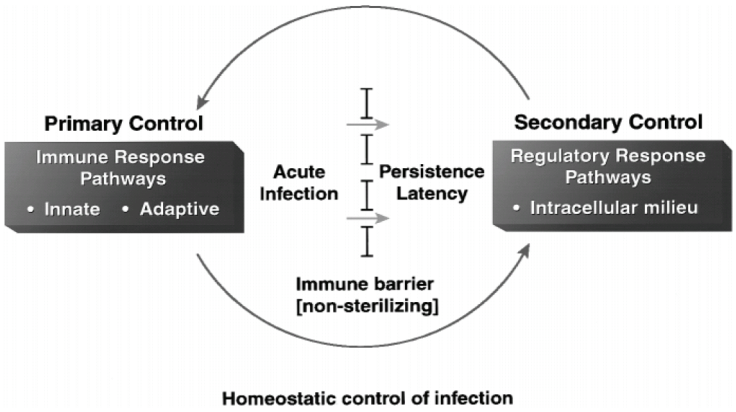

Figure 1. Homeostatic control of infection (Ghazal et al., 2000a).
Primary control is exerted by immune responses and dependency on host factors provides a secondary level of hierarchical control at the cellular level.

Microorganisms exist in a highly dynamic state of equilibrium (homeostasis) with their hosts, in which both immune and non-immune pathways contribute to control. Pathogen survival, in virus infections results in viral clearance and/or asymptomatic homeostasis. In this light, an infection can be viewed as a highly complex interaction that takes place between two partners: the host and the pathogen. The host vs. pathogen interaction has provided the driving force for the reciprocal evolution of the immune system and the many pathogen strategies for host evasion.

The virus achieves a dynamic balance with the host immune response that ensures survival of both pathogen and host. Their study cannot merely reveal how viral mechanisms survive and cause disease, but also new aspects of immune responses. Therefore, the immune system is not completely efficient in clearing a virus during a primary infection. The residual population of virus may eventually be eliminated or, as is common for a number of viruses, adopt one of two viral survival strategies: The establishment of persistence or latency.

In addition to the innate and adaptive immune responses, a secondary level of control is employed by non-immune pathways that are associated with immunity and is connected to the obligate dependency of the virus on its host. This secondary stage of control plays an essential role in modulating infections at the cellular level. Essentially, three categories of regulatory response pathways provide the secondary level of control in the infected cell: the cell cycle, apoptosis, and the intra- and extracellular signalling pathways, all of which reflect changes in the intracellular milieu of the cell (Ghazal *et al.*, 2000b). Viral pathogenesis is varied, with each progression being characterized by distinct clinical manifestations and outcomes. These include: a) acute infection; b) latent

infection; c) persistent infection and d) slow virus infection. Acute viral infections are characterized by a comparatively rapid incubation time, production of large amounts of infectious particles, and resolution either by complete viral clearance or death of the host. In the human respiratory tract for example, viruses can produce a spectrum of acute infections, ranging from the common cold and acute bronchiolitis to pneumonia (influenza virus).

In contrast, latent and persistent infections result from failure of the host to completely clear the virus after acute infection, suggesting that the virus has evolved a mechanism for immune evasion. In latent infections, infectious virus “disappear” after the acute phase, but can reappear at a later time. This involves incomplete expression of viral genes during periods of latency or quiescence and production of fully assembled, replicating viral particles during episodes of reactivation or recurrence. Common stimuli of reactivation include an acquired immunodeficiency (i.e., organ transplant recipients on immuno suppressive therapy) or stress.

Many DNA viruses produce latent infections. Varicella zoster virus exhibits the prototypical latent infection. Persistent infection is characterized by continuous viral replication (and therefore, persistence of infectious virus), but usually at a lower level than is observed during acute infection. This type of infection is also well described for RNA viruses such as measles and lymphocytic choriomeningitis. Chronic infection differs from latent viral infections in that viral proteins, which might stimulate host immune and inflammatory responses, are produced as a result of complete viral replication. Slow virus infections, such as rapidly progressive dementias caused by prions (proteinaceous infectious particles), have only been described to affect the central nervous system (Alcami & Koszinowski, 2000) .

The protection from a vast range of potential pathogens encountered during the lifetime of an individual is achieved through the highly coordinated action of the innate and adaptive arms of the immune system. The innate branch of the immune system provides a rapid, and relatively nonspecific, initial response to pathogens. In contrast, the adaptive branch is required for presentation of the four classic features of an immune response, namely specificity, memory, diversity, and self-non-self discrimination.

The development of the adaptive arm of the immune system is dependent on the tightly regulated activation of B and T lymphocytes in response to antigenic stimulation. When a vertebrate animal is infected by a pathogen, the immediate response is a non-clonal innate response. Meanwhile an adaptive immune response is induced.

Notably, the innate immune system also has a key role in the activation and modulation of the adaptive immune system as merely the recognition of an antigen by a lymphocyte is not sufficient to initiate an immune response and, in fact, can induce suppression of T cells.

A common practice to efficiently induce adaptive responses is co-injection of complete Freund's adjuvant. This is a mixture of killed mycobacteria in oil, which was appropriately described by Janeway (1989) as "*the immunologist's dirty little secret.*" Adjuvants are thought to trigger the innate immune system, which subsequently provides co-stimulatory signals required to stimulate the adaptive immune system (Fearon, 1997; Janeway, 1989). The context of an antigen (e.g. its localization, the presence of conserved pathogen-specific structures, and any tissue damage) is a determinant factor controlling the direction of an acquired immune response. Janeway suggested that the innate signals allow the vertebrate immune system to distinguish between infectious non-self and non infectious self molecules (Janeway, 1992).

An illustrative example of the importance of the context of antigens in the induction of adaptive responses was given by an experiment where viral proteins were converted into self-antigens by inserting their genes into the germ line of mice and they failed to provoke autoimmunity. The adaptive immune system did not "tolerate" the viral antigens, but refrained from responding because the antigens were presented in a non-inflammatory context. Only when the mice were subsequently infected with the live virus (Ohashi *et al.*, 1991; Oldstone *et al.*, 1991) initiation of adaptive immunity resulted from an antigen-specific release of nonspecific effectors mechanisms (Janeway, 1989). Specific binding between antibody and antigen, for example, triggers the complement cascade and attracts phagocytic cells and killer cells. Similarly, specific antigen recognition by T cells can lead to the release of nonspecific cytotoxic molecules. Thus, the vertebrate immune system combines the evolutionary wisdom of the innate immune system with the large diversity of the adaptive system.

The need for innate signals in the induction of adaptive responses, however, would allow pathogens to evade the adaptive immune response by manipulating the innate response. The flexibility supplied by the random generation of lymphocytes is thus vulnerable through its requirement for innate signals (Janeway, 1992; Medzhitov *et al.*, 1997). This dependence of the adaptive immune system on evolutionarily conserved innate signals raises an evolutionary problem. It is often argued that the adaptive immune system evolved to cope with rapidly coevolving pathogens. The clonal distribution of randomly rearranged lymphocyte receptors provides a high flexibility of possible responses,

perhaps enabling the adaptive immune system to adapt more quickly to coevolving pathogens than the innate immune system can. However, if an adaptive immune response depends strictly on the innate immune system, then pathogen evasion of an innate response implies the possible subsequent evasion of an adaptive immune response.

Viruses have indeed been shown to interfere with the innate immune system by producing proteins (e.g. soluble cytokine receptors or proteins that regulate antigen presentation) that put the immune system "on the wrong track". Some years ago, a commentary in Science (Barinaga, 1992) announced that viruses engage in "Star Wars" strategies against the immune system. Some of the viral invaders make receptors ("viroceptors") that mimic normal cellular receptors and so can sequester and inactivate molecules critical to the function of adaptive immunity (Goodman, 2004).

Since then, numerous other viral subterfuges for evading or subverting host defence mechanisms have been described and viruses are now known to employ an extraordinary spectrum of proteins to target immune molecules of the host cells (Alcami & Koszinowski, 2000; Engel & Angulo, 2012; Finlay & McFadden, 2006; Vossen *et al.*, 2002). One particularly effective host defence for the infected cell is to self-destruct by programmed cell death, and in fact, cell death is triggered by infection with a wide variety of viruses. Many viruses use specific proteins to suppress apoptosis in order to extend the life of their cellular host and thus provide the necessary time for their replication (Aubert & Jerome, 2003).

1.3 The innate arm of the immune system

The innate immune system is the first line of host defence against pathogens and is largely mediated by phagocytes including macrophages and dendritic cells (DC). The innate immune system is not completely unspecific, as it was initially thought, since it does discriminate between self and a variety of pathogens. To do this, the innate immune system uses a limited number of germ line-encoded pattern-recognition receptors (PRRs). The principal mechanisms of innate immunity are natural antibodies, complement mediated mechanisms, inflammation and the induction of apoptosis, chemokines and cytokines.

The immediate response to viral infection relies on PRRs, most prominently the Toll-like receptors (TLRs) and the RNA helicases RIG-I and MDA-5, as well as double stranded RNA-dependent protein kinase (PKR) and the DNA receptor, DAI. These PRRs recognize

pathogen-associated molecular patterns (PAMPs) such as viral proteins and nucleic acids. The engagement of these receptors then initiates intracellular signalling cascades which ultimately cause the activation of transcription factors and the expression of type I interferons and pro-inflammatory cytokines (Vossen *et al.*, 2002). This innate response establishes an anti-viral state in the infected cell and its neighbours and alerts immune cells to the danger. In order to establish a productive infection, viruses need to overcome this initial anti-viral response.

For the early control of virus infections, in particular acute infections, the interferon response is of paramount importance for most viruses and is a particular focus of this thesis. Viruses have evolved several molecular strategies to act over different levels on the signalling pathway to downregulate the IFN system. The exact strategy used by a virus will presumably depend on the biology of the infection and will be a major factor that will influence the pathogenesis of that virus infection (Randall & Goodbourn, 2008). Often viral antagonists are multifunctional proteins that interact with multiple host components, in this manner increasing the efficiency of their host evasion and also allowing the virus to influence diverse biological processes in infected cells. The size of viral genomes contrasts with the number of mammalian genes dedicated to host defence mechanisms, providing an additional selective pressure for the evolution of such viral multifunctional proteins (Haller *et al.*, 2006; Vossen *et al.*, 2002).

1.3.1 The Toll-like receptors (TLRs) family

Toll was initially described as a type-I transmembrane receptor with an important role in defence against fungi and Gram-positive bacteria in Drosophila melanogaster. The extracellular domain of Toll contains leucine-rich repeats (LRR), whereas the intracellular tail of the receptor shares striking homology with the intracellular domain of interleukin-1 (IL-1) receptor type I, which is designated the Toll–IL-1R (TIR) domain **(Figure 2)**. The initial data suggested that Toll is an important component of the antimicrobial defence of Drosophila, and the suspicion that mammalian homologues might have similar functions has proved to be well founded. To date, 13 mammalian TLRs have been identified, and they all share similarities in their extracellular and intracellular domains. The molecular basis of their intracellular signalling depends on the conserved part of their TIR domain (Brikos & O'Neill, 2008; Kang & Lee, 2011; Watters *et al.*, 2007).

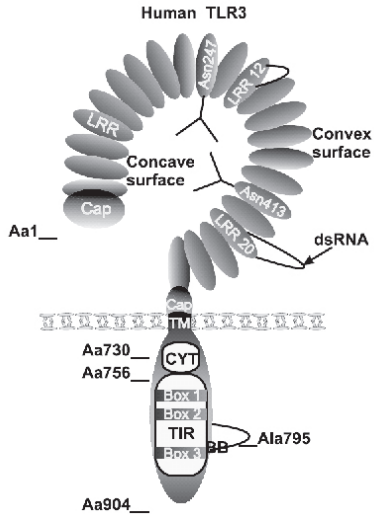

Figure 2. Schematic structure of an human TLR (Vercammen et al., 2008).

TLRs are activated in response to a large spectrum of conserved structures called pathogen associated molecular patterns (PAMPs), ranging from bacterial and viral components to fungal and protozoan molecules, for example, bacterial lipoproteins, lipoteichoic acid and zymosan are recognized by TLR2; double-stranded RNA by TLR3; lipopolysaccharide (LPS) and heat-shock proteins by TLR4; flagellin by TLR5; single-stranded RNA by TLR7 and TLR8; and CpG motifs of bacterial DNA by TLR9. A vast number of ligands have been identified that are specifically recognized by different TLRs. A multitude of studies have reported additional microbial ligands for TLRs, as summarized in many reviews (Demengeot *et al.*, 2006; Gay & Gangloff, 2007; Kang & Lee, 2011; Uematsu & Akira, 2006). The ability not only to respond appropriately but also to self-regulate host response to invading pathogens is vital to the capacity to mount an appropriate primary immune response. After recognition, a cascade of intracellular signalling events is activated which culminates in the induction of pro-inflammatory cytokines.

1.3.2 Dimerization as a requirement for signaling by TLRs

The binding of agonistic PAMP ligands induces dimerization of the ectodomains of the various TLRs, forming strikingly similar structures (“m”-shaped complexes). The dimerization of the extracellular domains results in the intracellular TIR domains, and this initiates signalling by recruiting intracellular adaptor dimerization of proteins (Jin & Lee,

2008). In addition, heterodimerization is reported to be responsible for differential recognition of PAMPs, and this is apparent in the distinction of di- and tri-acylated lipopeptides by TLR2–TLR1 and TLR2–TLR6 heterodimers, respectively.

There are five adapters identified so far, named **a)** Myeloid differentiation primary response protein 88 (MyD88), **b)** MyD88-adapter like (Mal) or TIR domain-containing adapter (TIRAP), **c)** TIR domain-containing adapter inducing interferon-beta (TRIF) or TIR domain-containing adapter molecule-1 (TICAM-1), **d)** TRIF-related adapter molecule (TRAM) or TICAM-2, and **e)** sterile alpha and HEAT-Armadillo motifs (SARM) (Kenny & O'Neill, 2008;). The first four play a fundamental role in TLR-signalling, defining which pathways will be activated, depending on which of these adapters will be recruited by each TLR. Among these adapter proteins MyD88 and TRIF are now considered as the signalling ones and hence the TLR pathways can be categorized as MyD88-dependent and TRIF-dependent (O'Neill & Bowie, 2007).

1.3.3 Signaling Pathways

TLRs initiate shared and distinct signalling pathways **(Figure 3)** by recruiting different combinations of TIR domain-containing adaptor molecules: MyD88 is used by all TLRs except TLR3; TIRAP is used by TLR2 and TLR4; TRIF is used by TLR3 and TLR4; and TRAM is used only by TLR4 .

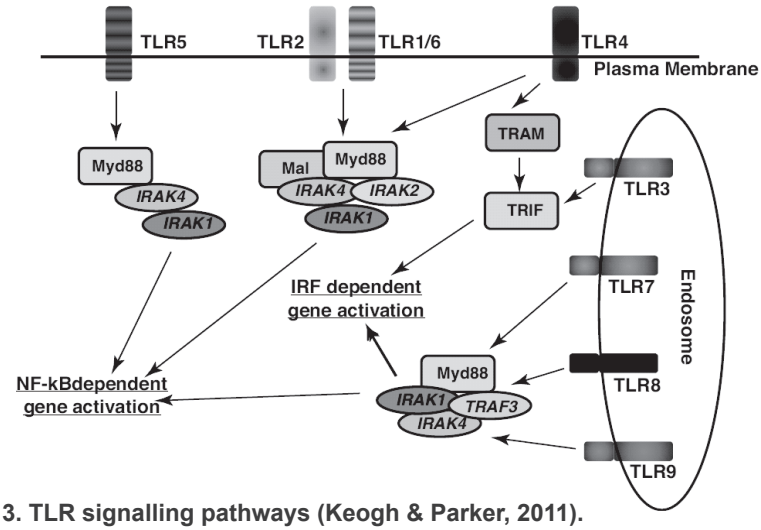

Figure 3. TLR signalling pathways (Keogh & Parker, 2011).
Upon activation by ligands, TLRs recruit TIR adapter proteins MyD88, Mal, TRAM and TRIF, which leads to recruitment of IRAKs and ultimately the induction of NFkB-dependent genes, including TNFα, IL-12, IL-6 and IL-8. TLR3 and TLR4 also signal via TRAM and TRIF leading to IRF3-dependent gene expression. In addition, IRF7 is activated downstream of TLR7, TLR8 and TLR9, which leads to IRF7-dependent gene expression, including IFN-β and IFN-inducible genes.

Interestingly, TLR4 induces two distinct signalling pathways controlled by the TIRAP-MyD88 and TRAM-TRIF pairs of adaptor proteins, which elicit the production of pro-inflammatory cytokines and type I interferon, respectively. TLR4 activated these two signalling pathways sequentially in a process organized around endocytosis of the TLR4 complex. TLR4 first induces TIRAP-MyD88 signalling at the plasma membrane and is then endocytosed and then activates TRAM-TRIF signalling from early intracellular endosomes (Kagan *et al.*, 2008). These signalling pathways activate the transcription factors nuclear factor NFκB and activator protein-1 (AP-1), which is common to all.

1.3.4 The TLR3 signaling pathway regulation

The signalling pathways of TLR3 are initiated by the sole recruitment of TRIF among TIR adaptors, which then activates the TRIF-dependent pathway, leading to the subsequent production of inflammatory cytokines and type-I IFNs, and to the up-regulation of co-stimulatory molecules **(Figure 4)**. TRIF is critical in mediating TLR3 signalling and is emphasized by the fact that it is targeted for immune evasion by some viral proteins **(Figure 5)** (Vercammen *et al.*, 2008).

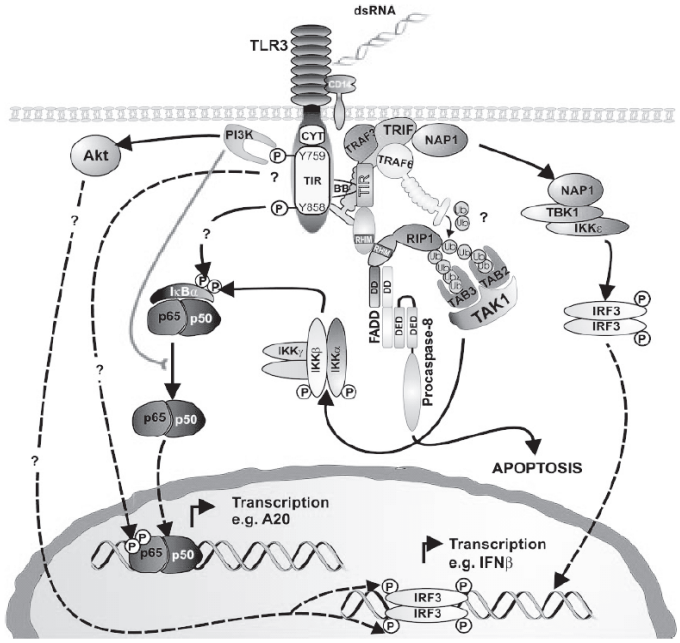

Figure 4. TLR3 signalling pathways (Vercammen et al., 2008).
Binding of dsRNA to the TLR3-CD14 complex induces the activation of several intracellular signalling pathways. The activation of NFkB and IRF3 is achieved by two different signalling branches originating from the TLR3 adaptor molecule .

TLR3 signalling is potentially harmful or even fatal for the host cell. Sustained TLR3 activation is associated with the overproduction of pro-inflammatory cytokines and can result in systemic inflammatory response syndrome.

In addition, excessive TLR3 expression or triggering is associated with several inflammatory diseases, such as inflammation-associated myopathies, lupus nephritis, West Nile virus-driven central nervous system inflammation, and viral or autoimmune liver disease. It is therefore not surprising that mammalian cells have also evolved several mechanisms for controlling TLR3-mediated responses. For example, Endogenous negative regulators interact with TRIF, such as the protein inhibitor of activated signal transducers and the activators of transcription (PIASy), TNF receptor-associated factor 1 (TRAF1) and the TIR motif-containing protein (SARM), A20 protein, and TNF receptor-associated factor 4 (TRAF4). However, these proteins inhibit NFkB as well as IRF3 activation. PIASy is a member of the SUMO-ligase family that also interacts with IRF3 and IRF7. Although this protein inhibits TRIF-induced NFkB and IRF3 activation, it has no effect on TRIF-induced apoptosis (Zhang *et al.*, 2004). TRAF1 is an inducible protein that binds to the TIR domain of TRIF and is cleaved by a TRIF-activated caspase. Because caspase inhibition or the expression of a noncleavable TRAF1 mutant abolishes the inhibitory effect of TRAF1, it has been suggested that TRIF-induced cleavage of TRAF1 is essential for the inhibition of TRIF signalling (Su *et al.*, 2006). The TIR-containing protein SARM also associates with the TIR domain of TRIF and is a broad inhibitor of TRIF-induced cytokine and chemokine production (Carty *et al.*, 2006).

A20 is a deubiquitinating enzyme that is induced by several stimuli, including dsRNA and Sendai virus infection. A20 has been shown to co-precipitate with TRIF and to inhibit TLR3-mediated NFkB and IRF3 activation. However, its deubiquitinating activity does not seem to be required for the inhibition of TRIF signalling (Wang *et al.*, 2004). Additionally, A20 has been shown to deubiquitinate RIP1, TRAF6, and IKK in the TNF and TLR4 signalling pathway to NFkB, suggesting that these signalling proteins might also be targeted in the TLR3 signalling pathway to NFkB. Furthermore, A20 also co-precipitates with TBK1 and IKKε and inhibits IRF3 phosphorylation and dimerization following the engagement of TLR3 (Saitoh *et al.*, 2005). Finally, TRAF4 is another inducible protein that also physically interacts with TRIF and TRAF6 and counteracts their function (Takeshita *et al.*, 2005)

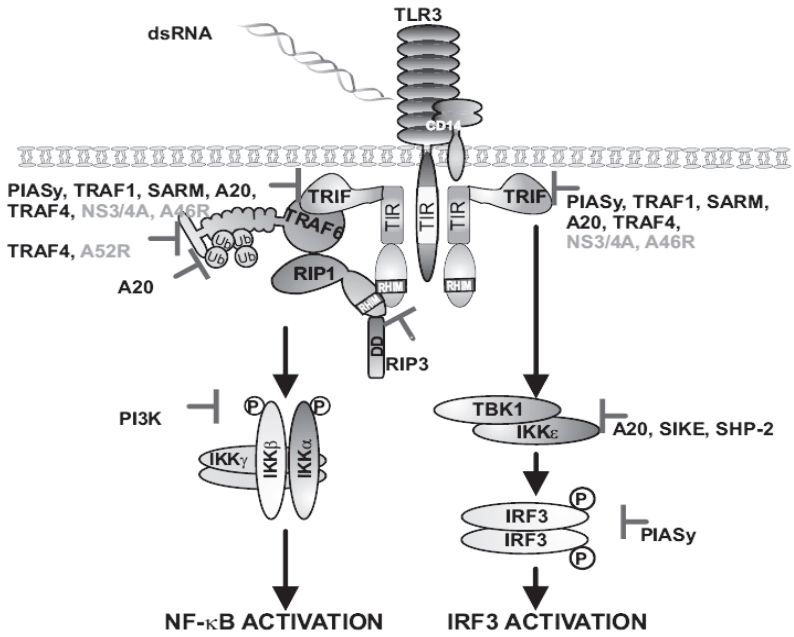

Figure 5. Endogenous and viral inhibitors of TLR3-mediated NFkB or IRF3 activation (Vercammen *et al.*, 2008).
Most known inhibitors interfere with the function of TRIF, either by interacting with TRIF (PIASy, TRAF1, SARM, A20, TRAF4, and the vaccinia virus protein A46R) or by degrading TRIF (hepatitis C virus protease NS3/4A). Other inhibitors interact with TRAF6 (TRAF4, A20, and vaccinia virus protein A52R), RIP1 (RIP3), TBK1/IKKε (A20, SIKE, and SHP-2), or IRF3 (PIASy).

In contrast to the above-described inhibitors that inhibit both NFkB and IRF3 activation, suppressor of IKKε (SIKE) interferes uniquely with TLR3-triggered IRF3 activation. Under physiological conditions, SIKE is associated with TBK1 and dissociates upon TLR3 stimulation. The over expression of SIKE disrupts the interactions of IKKε or TBK1 with TRIF and IRF3, without affecting the interactions of TRIF with TRAF6 and RIP1. Consistently, the overexpression of SIKE inhibits virus- and TLR3-triggered IRF3 but not NFkB activation (Huang *et al.*, 2005).
Due to the need for phospho-tyrosine residues in the TIR domain of TLR3, one might expect that an alternative way of interfering with TLR3 signalling is dephosphorylation by a tyrosine phosphatase. In this context, SH2-containing protein tyrosine phosphatase 2 (SHP-2) was recently reported to inhibit TLR3-activated IFN-β production. However, this seems to occur by a phosphatase activity-independent mechanism, in which SHP-2 interacts with the kinase domain of TBK1 to inhibit its activity (An *et al.*, 2006).
Although it is surprising how many different endogenous or viral proteins and mechanisms have evolve to negatively regulate

TLR3 signalling **(Figure 5)**, this complexity also underscores the importance of the process. The diversity of NFkB and IRF inhibitory proteins may have evolved to establish a redundant system in which one negative-feedback regulator can compensate for the loss or failure of others. Moreover, specific regulatory proteins might change the balance between NFkB and IRF3 activation. Most likely, the role of specific negative regulatory proteins also depends on the cell type or the cell context.

1.3.5 Innate antiviral responses

Each TLR has common effects, such as inflammatory cytokine and chemokine induction or upregulation of costimulatory molecule expression, but also has its specific function. They activate interferon regulatory factor 3 (IRF3) and/or IRF7, leading to the production of type I IFNs such as IFNα and IFNβ (Colonna, 2007). Activation of the IRFs is crucial for the induction of type I IFNs, and although NFkB also contributes to IFN induction, it is mainly required for the induction of pro-inflammatory cytokines. Type I IFNs can induce an antiviral state in most cells and, in addition, have diverse functions in the development of adaptive immunity (Barnes *et al.*, 2002; Haller *et al.*, 2006). IRF together with NFkB family of transcription factors are the two key transcription factors that are activated during an antiviral immune response. TLR3- and TLR4-induced IRF activation is mediated by TRIF, whereas MyD88 is sufficient for IRF activation induced by TLR7, 8 and 9 (Colonna, 2007). TLR signalling to NFkB proceeds mainly via MyD88, the IRAKs and TRAF-6.

The signalling pathway emanating from RIG-I is less well defined, but it involves a mitochondrial adaptor molecule called IPS-1 (IFN-β promoter stimulator) also called MAVS (mitochondrial antiviral signalling), Cardif [CARD (caspase recruitment domain) adaptor inducing IFNβ] or VISA (virus induced signalling activator) for activation of both NFkB and IRF-3. Even though their upstream signalling pathways are distinct the signalling pathways elicited by the different classes of PRRs, converge at the level of the IKK [IκB (inhibitory kB) kinase] complex (containing IKK-α, IKK-β and IKK-γ) for activation of IRF-3 and IRF-7.

1.3.6 Role of TLRs in macrophages

Toll-Like Receptors are very important for a rapid activation of the innate immune system, especially for the efficient function of macrophages. The latter are very important because they are the scavengers of the immune system, producers of immunomodulatory

molecules and, for this thesis, because they are the main target cells of African Swine Fever Virus (ASFV). Due to their heterogeneity, macrophages have the ability to reside in many different tissues of the body. Their function is to 'reside' in those tissues and remove apoptotic and necrotic cells, as well as any invading pathogens. To do this, macrophages express surface receptors that bind to apoptotic cells and invading pathogens, leading to their internalization in a process known as phagocytosis. In addition, it is crucially important that, in response to pathogens, macrophages are able to mount an appropriate immune response to further aid in the fight against infection. The macrophage contribution to the immune response can be divided into an immediate innate response through the detection and engulfment of invading pathogens and the secretion of immunomodulatory molecules, and into acquired immune response through antigenic presentation, thus initiating T cell responses (Banerjee & Gerondakis, 2007).

The major receptors for detection of invading pathogens are the well-known Toll-like receptors (TLRs). These are expressed in a cell-specific way, varying greatly from cell-to-cell. Unlike other cells that express only some TLRs, macrophages express most of the known TLRs. The fact that most of the TLRs are expressed by macrophages stresses the importance of these receptors for the function of these cells. Not only are they essential for the immediate detection of invading pathogens, thereby initiating an inflammatory response, but also they are important for each of the stages of phagocytosis, ranging from engulfment of invading pathogens, to antigen processing and presentation of antigenic peptides. In addition, TLR signalling is also required for the up-regulation of cytokines, MHC molecules and co-stimulatory molecules that are needed to mount an appropriate adaptive T cell response.

Furthermore, to some extent TLR expression is specie specific, differing among species. For example, porcine alveolar macrophages express TLR3 (Chaung *et al.*, 2010; Liu *et al.*, 2009; Sang *et al.*, 2008), whereas bovine alveolar macrophages evidently do not (Werling *et al.*, 2006). Moreover, differences exist between and within species in regards to TLR3 regulation. For example, TLR3 expression is induced by LPS in murine macrophages, but not in human macrophages (Alexopoulou *et al.*, 2001). In murine macrophages TLR3 upregulation involves autocrine/paracrine IFN-β, whereas in human macrophages the IFN-β induced upregulation of TLR3 is blocked by pre-treatment with LPS (Heinz *et al.*, 2003). Mice fail to express TLR10, however they express additional TLRs such as TLR11, TLR12 and TLR13 which are absent in humans (Beutler, 2004).

Phagocytosis by macrophages is essential for the removal of apoptotic and necrotic cells, as well as for the ingestion and processing of invading pathogens. These results in the death of the pathogen and the presentation of antigen peptides derived from pathogenic proteins and incorporated into MHC class II of the Antigen Presenting Cell (APC). The MHCII-peptide complex is recognized by CD4+ T cells, resulting in the activation of the adaptive immune responses. As Phagocytosis of pathogens often goes together with inflammatory responses and the inflammatory response is driven by TLRs, it was suggested that phagocytosis and TLR activation could be functionally linked (Underhill & Gantner, 2004).
Evidence that TLRs play a role in phagocytosis come from a study that show that TLR-1, TLR-6 and TLR-2 were recruited to phagosomes containing bacterial components in all stages of phagocytosis, but also to phagosomes containing IgG-opsonized erythrocytes that do not display any microbial components. This suggests that TLRs are recruited to phagosomes, where they interact with pathogen PAMPs, leading an inflammatory response (Ozinsky *et al.*, 2000). Stimulation of mammalian cells transfected with porcine TLR3 and TLR7-constructs with their respective agonists, poly (I:C) and imiquimod (R837), and adenovirus provoked activation of interferon regulatory factors (IRFs) providing molecular and functional information about the role of TLR3 and TLR7 in mediating immune protection against porcine viral diseases (Sang *et al.*, 2008).

1.3.7 Mechanisms to manipulate PPRs

Viruses have also evolved a range of mechanisms to interfere with the signalling pathways elicited in response to their detection by the innate immune system. Some viral proteins specifically target certain PRRs or their downstream signalling pathways, while others have a broader range of action and interfere with NFkB and/or IRF activation more generally.
Three major TLR-mediated escape mechanisms have been identified to-date: a) TLR2-mediated immunosuppression, due to either premature or biased anti-inflammatory effects; b) TLR-mediated induction of viral replication; c) Prevention of TLR recognition, which inhibition of the IFN response due to stimulation of TLR3 being of particular relevance in virus infections.

a) TLR2-mediated immunosuppression, due to either premature or biased anti-inflammatory effects:
In contrast of TLR4, TLR2 signals are strong mediators of anti-inflammatory effects and are needed during the recovery

phase of infection for the reversal of the inflammatory process. Immunosuppression induced by TLR2 is due to either an exaggeration or a premature activation of the normal anti-inflammatory effects of TLR stimulation. The first study that investigated the differential effects of TLR2 and TLR4 stimulation on dendritic cells, reported the failure of TLR2 ligands to induce the release of IL-12 and interferon (IFN-γ), thus favouring a Th2-type response (Hirschfeld *et al.*, 2001). As mentioned above, microorganisms have developed strategies to either block or avoid their recognition by TLRs and hence the subsequent activation of innate defences. For example, *Treponema* inhibits cell activation induced by several TLRs (TLR3, TLR4 and TLR9) by blocking the function of LPS-binding protein and CD14 (Re & Strominger, 2001).

b) TLR-mediated induction of viral replication:

A different strategy for escaping TLR recognition is used by the fungus Aspergillus fumigates. It evades immune recognition by germinating hyphae that are not recognized by TLR4 recognition, but are recognized by the TLR2, thus shifting the balance towards a permissive IL-10 and Th_2-type profile (Netea *et al.*, 2003). Several bacterial pathogens have also modified the structure of particular PAMPs to avoid recognition by TLR4 or TLR5; pathogens, such as *Porphyromonas gingivalis* or *Leptospira*, have LPS structures (normally recognized by TLR4) that only interact with TLR2 (Hirschfeld *et al.*, 2001; Werts *et al.*, 2001), whereas flagellin of *Helicobacter pylori* is not properly recognized by TLR5, permitting the survival of the bacteria without loss of virulence (Gewirtz *et al.*, 2004).

A particular form of immune evasion is represented by stimulation of viral replication through TLR activation and is demonstrated specifically by retroviruses. In this respect, signalling through TLR2, TLR4 and TLR9 significantly enhances human immunodeficiency virus (HIV)-1 replication in either mast cells (Sundstrom *et al.*, 2004) or transgenic mice. During coinfection with mycobacteria and HIV-1, HIV-1 expression is potentiated by mycobacteria through TLR2 stimulation (Báfica *et al.*, 2003). Another retrovirus, the Mouse Mammary Tumour Virus (MMTV), persists indefinitely in C3H/HeN mice, but not in the TLR4-defective C3H/HeJ mice. The immune escape of MMTV by persistent infection is mediated by TLR4-triggered production of the immunosuppressive cytokine IL-10 (Jude *et al.*, 2003).

c) Prevention of TLR recognition, which inhibition of the IFN response due to stimulation of TLR3 being of particular relevance in virus infections.

Recently, viral proteins have been described which prevent RNA detection through RIG-I and MDA-5: the *Paramyxovirus V* proteins, which bind to MDA-5, and Influenza Virus NS1, which binds to RIG-I. Other proteins such as Ebola virus VP35 bind to the dsRNA and thereby prevent detection by RIG-I and also PKR. Hepatitis C virus expresses the protease NS3/4a, which cleaves TRIF and the RLH (RIG-like helicase) adaptor IPS-1 and thereby specifically targets and evades TLR3- and TLR4- as well as RLH-signalling.

All these examples suggest that microorganisms manipulate specific PRR-mediated signals to escape from the host defence, either by down-modulation of leukocyte function, or amplification of viral replication. Definitely virus mechanisms for manipulation of TLR mediated responses provide a route towards better antiviral strategies and vaccines.

Other examples for viral proteins that target specific PRR signalling pathways are the VACV (Vaccinia virus) TLR antagonists A52 and A46. The poxvirus protein A52R blocks activation of the pivotal transcription factor NFkB, which is induced by multiple TLRs (including TLR3) through association with IRAK2 and TRAF6 (Tumour Necrosis Factor Receptor-Associated Factor-6), two key proteins of the TLR intracellular signalling cascade, and interferes with the induction of pro-inflammatory cytokines (Harte *et al.*, 2003; Maloney *et al.*, 2005). A46 contains a TIR (Toll/IL-1 receptor) domain, interacts with the host TIR adaptors and thereby disrupts TLR signalling to NFkB and IRF-3 (Stack *et al.*, 2005). Apart from A46 and A52, VACV encodes a whole range of immunomodulatory proteins, including several NFkB inhibitors, such as N1L K1 (Shisler & Jin, 2004) and M2 (Gedey *et al.*, 2006) **(Figure 6).**

All these examples suggest that microorganisms manipulate specific PRR-mediated signals to escape from the host defence, either by down-modulation of leukocyte function, or amplification of viral replication. Definitely virus mechanisms for manipulation of TLR mediated responses provide a route towards better antiviral strategies and vaccines.

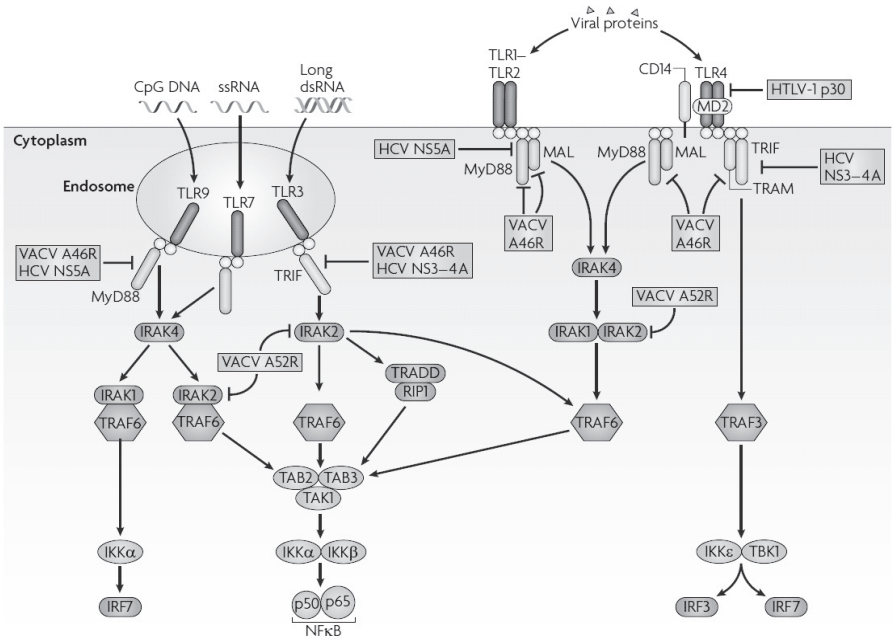

Figure 6. Viral evasion and subversion of Toll-like receptor signalling (Bowie & Unterholzner, 2008).

Following activation; recruit the adaptor proteins MyD88, TRIF, MAL and TRAM, as indicated. These then triggers signalling cascades involving IRAK and TRAF proteins, which finally converge at the activation of the IKK family members and TBK1.

The vaccinia virus protein A46R sequesters all these adaptor proteins, whereas the hepatitis C virus protein NS5A selectively binds MyD88 and the HCV NS3–4A protease cleaves TRIF. Human T-cell leukaemia virus type 1 protein p30 acts even further upstream by reducing the expression of TLR4. VACV A52R binds to and inhibits IRAK2, thereby affecting several TLR pathways that lead to NFkB activation .

1.4 African Swine Fever Virus (ASFV)

African Swine Fever Virus (ASFV) is able to survive in a vertebrate (pig) and an invertebrate (tick), which suggests that this virus may have evolved efficient “immune evasion” strategies in both of these two hosts. This, and the fact that in the pig the virus uniquely infects the central cell of the innate immune response, the macrophage, raises the possibility of Toll-like receptor (TLRs) signalling antagonists in the ASFV genome. Interfering with innate immune response is a common strategy in viruses for host evasion and escape of recognition by the immune system; thus avoiding being eliminated from the organism (De Oliveira *et al.*, 2011).

The principal event of pathogen recognition is the interaction of their PAMP’s with host PRR’s triggering the first steps of immune response. Various intracellular signalling pathways can operate between recognition of the pathogen and the ultimate cellular response.

The replication of ASFV in macrophages provides an opportunity for the virus to interfere with both the innate and de adaptive response to the infection by manipulating macrophage function. Different classes of ASFV encoded proteins which interfere with host defences have been identified (Chapman *et al.*, 2008; Dixon *et al.*, 2004). And many more are thought to inhibit this and others immune pathways.

1.4.1 Pathogenicity and epidemiology

The ASFV causes an acute haemorrhagic fever with high mortality rates in domestic pigs, and unapparent infection with transient, low viremic titers, persistent infections in its natural hosts, warthogs, bush pigs and soft ticks of the species *Ornithodoros*. The pathogenesis of ASFV in ticks (*O. porcinus porcinus*) is characterized by a low infectious dose, lifelong infection, efficient transmission to both pigs and ticks, and low mortality until after the first oviposition. Thus ticks probably constitute the most important natural vector of ASFV, although both the mammalian and tick hosts are probably required for the maintenance of ASFV in the sylvatic cycle (Kleiboeker & Scoles, 2001).

The disease is a problem for pig farming worldwide and leads to economic losses in affected countries. The main targets for virus replication are cells of monocyte/macrophage lineage and those expressing cell surface markers characteristic of intermediate/late stages of differentiation are permissive for virus replication (McCullough *et al.*, 1999). In the early stages of infection virus replication is seen primarily in macrophages and this is most likely the primary event leading to the haemorrhagic and lymphoid apoptotic pathology

(Oura *et al.*, 1998). In common with other viral haemorrhagic fevers, ASFV is characterized by damage to vascular endothelial cells and this contributes to vascular permeability. This is thought to be caused by factors released from virus-infected macrophages, although direct infection of endothelial cells may be involved. As infection of endothelial cells *in vitro* by ASFV leads to induction of apoptosis which could contribute to the destruction of vascular endothelial cells in infected pigs (Vallée *et al.*, 2001).

1.4.2 Virus transmission

Montgomery in Kenya first identified the disease in the 1920s following spread from infected wildlife. Subsequently it was established that ASFV infection has been present over a very long period in Eastern and Southern Africa in warthogs (*Phacochoerus aethiopicus*), bush pigs (*Potamochoerus porcus*) and a species of soft tick (*Ornithodoros porcinus*). These wildlife hosts can be persistently infected over long time periods without showing disease signs. Ticks become infected by feeding on warthogs and young warthogs develop a sufficiently high viraemia to infect ticks. Virus can be transmitted between ticks transadially, transovarially and transexually from male to female. Infected ticks transmit virus to warthogs on which they feed. Direct transmission between warthogs is thought to be infrequent and thus the tick vector plays an important role in the sylvatic cycle (Kleiboeker & Scoles, 2001).

The virus is very well adapted to its hosts, probably reflecting a long-term evolution and adaption in this transmission cycle. Once introduced into the domestic pig population, the virus can be transmitted directly between pigs without the requirement of the tick vector, although the tick may provide an important reservoir of virus if it present in pig houses. Spread of ASFV to most sub-Saharan African countries has occurred and it was also introduced to Portugal in 1957 and 1960. The disease remained endemic in Spain and Portugal until the mid-1990s. In southern Spain and Portugal the tick species Ornithodoros erraticus acted as a vector for the virus contributing to the difficulties in disease eradication. ASFV is thought to have spread to other European countries and to Brazil and the Caribbean from Spain and Portugal. Except for Sardinia outside Africa ASFV has now been eradicated (Costard *et al.*, 2009). However recently, ASFV has entered countries of the old Soviet Union and is considered to be out of control (Chapman *et al.*, 2011)

1.4.3 Virus Structure, Entry, Replication Cycle and Assembly

ASFV is a large, enveloped, icosahedral double-stranded DNA virus and is the only recognized member of the Asfarviridae family (Dixon *et al.*, 2012). The virus shares similarities in its genome organization with other large DNA virus families, which replicate at least partially in the cytoplasm. These virus families may share a common ancestor. This virus superfamily, named the nucleo-cytoplasmic large DNA virus family (NCLDV), also includes the *Poxviridae, Iridoviridae, Phycodnaviridae* and *Mimiviridae* (Lyer *et al.*, 2006). Also the replication strategy of ASFV is similar to the *Poxviridae* (Rodríguez & Salas, 2012).

1.4.4 Virus encoded proteins

The ASFV genome encodes about 150 proteins, including structural proteins, enzymes and factors required for replication and transcription of the virus genome. There are in addition, many other proteins which have evolved for manipulation of host cell biology and immune response and which have a role in facilitating virus survival, persistence and transmission.

1.4.5 ASFV proteins involved in host immune evasion

Since ASFV shares a similar replication strategy with poxviruses, it has been predicted that the number of proteins involved in virus replication is similar in the two viruses. A core of 90 genes is conserved between different genera of the Chordopoxvirinae subfamily, suggesting that this represents the minimal number of genes required for replication (Upton *et al.*, 2003).Thus, 70 to 85 ASFV ORFs may encode proteins that are not essential for replication. Many of the remaining plays an important role in host manipulation. These include a number of proteins, which help the virus to evade host defences and persist in the host. By interfering with macrophage function, the virus could diminish both the initial innate response to infection as well as the later acquired immune response. The identified ASFV virus proteins with known roles, up to date, in evading host defences can manipulated the system into several ways (Dixon *et al.*, 2012) 1) ASFV proteins that interfere with activation of transcription factors of genes encoding immunomodulatory proteins; 2) ASFV adhesion proteins, which can modulate interactions of infected cells or extracellular virions with the extracellular environment; 3) ASFV proteins that inhibit apoptosis and can thus prolong survival of infected cells and facilitate virus replication; 4) ASFV Proteins that inhibit type I interferon induction and responses or use TLR-based escape mechanisms.

1.5 The adaptive arm of the immune system

The key players in the adaptive immune response are T and B-lymphocytes. The T lymphocytes that mature in the thymus are activated through a system known as antigen presentation. In this process, the antigen-presenting cell (APC) activates the T or B cell by presenting a peptide derived from a protein antigen. Antigen presentation is achieved via two different molecules: major histocompatibility complex (MHC) class-I molecules and MHC class-II molecules. MHC class-I molecules are used by all of the body's cells to display proteins from inside the cell, which have been degraded to peptides and then incorporated into the MHC-I molecule. This type of antigen presentation is principally used to signal cytotoxic T cells of the immune system to kill infected cells. In contrast, MHC II molecules are used by the antigen presenting cells of the immune system to display proteins brought in from the extracellular environment and causes the activation and proliferation of naive helper T cells. Specialized antigen presenting cells, particularly dendritic cells, are instrumental in presentation using MHC-II molecules.

All T cells have a TCR, composed of two different polypeptide chains that recognize a specific antigen. While approximately 5% of circulating T cells contain a γδ receptor and do not express either CD4 or CD8 molecules, the vast majority of mature T cells, contains a αβ-TCR and express CD4 or CD8 on their cell surface (Marrack *et al.*, 1983). These cells recognize a small peptide antigen presented by an MHC molecule on the surface of an infected cell (CD8+ T cells) or activated immune cell (CD4+ T cells). Within the two classes of αβ T cells, helper T cells express the CD4 molecule and recognize exogenous antigen presented on an MHC-II molecule, and cytotoxic T cells express the CD8 molecule and recognize endogenous antigen presented on an MHC-I molecule.

The CD4 and CD8 molecules act as co-receptors and signalling molecules, assisting in antigen recognition and guiding the cell which actions to take in response to activation. Following antigen recognition, the cell's T cell receptors cluster together, or cross-link, to allow the cell to reach the signalling threshold necessary to activate the cell. This cross-linking often occurs through signalling aggregates in the cell surface called lipid rafts, which bring together TCRs and other associated signalling molecules in order to generate and amplify a signal. A T cell activated through its cross-linked TCRs will proliferate and up regulate growth factor receptors on its surface. This activation also requires additional co-stimulatory molecules, such as B7 on an antigen-presenting cell (Janes *et al.*, 1999).

CD4+ T helper cells help to activate B cells and other T cells by

secreting cytokines. A naïve helper T cell (Th_0) when encounter antigens in secondary lymphoid organs usually secretes only IL-2, but upon activation, it will generally differentiate depending on the signals it receives from environment they are capable of differentiating into inflammatory Th_1 cells, helper Th_2 cells or pathogenic Th_{17} cells, which are distinguished by the cytokines they produce. Th1 cell usually secretes cytokines such as IL-1, IFN-γ, and TNF, activate macrophages and participate in the generation of cytotoxic lymphocytes, resulting in a cell-mediated immune response, Th_2 cell secretes IL-4, IL-5, IL-6 and IL-10, while Th_{17} cell secretes IL-17 and IL-22, pro-inflammatory cytokines that have been linked to the pathogenesis of autoimmune diseases and to immune responses to bacterial and fungal infections (Fang et al., 2010). Therefore, the immune response is directed to the type of response that is required to deal with the pathogen encountered cellular-mediated responses for intracellular pathogens or antibody responses for extracellular pathogens. Equally important, each subpopulation can exert inhibitory influences on the other. IFN-γ produced by Th_1 cells inhibits proliferation of Th_2 cells and differentiation of Th_{17} cells and IL-10 produced by Th_2 cells inhibits production of IFN-γ by Th_1 cells. In addition, IL-4 inhibits production of Th1 cells and differentiation of Th_{17} cells (Schmidt-Weber *et al.*, 2007).

The CD8+ T cells, or cytotoxic lymphocytes (CTLs) stop the spread of the infection by killing infected cells. Activation of CTLs requires antigen presentation via MHC-I molecule, as well as signals from helper T cells. Upon activation, and subsequent proliferation, the CD8+ T cells circulate in the bloodstream and recognize the appropriate infected cell by its display of pathogen peptide-MHC-I complexes. Recognition is then followed by killing of the infected cell through its perforin, which creates holes in the cell membrane, or FasL, which binds to a receptor on the cell surface, thus inducing programmed cell death (apoptosis) in infected cells. While the vast majority of effector cells disappear from the circulation following pathogen clearance ("activation induced cell death"), there remains a heterogeneous population of circulating or in peripheral tissues long-lived CD8+ memory T cells capable of mounting rapid recall responses on antigen reencounter (Zhang & Bevan, 2011).

All T cells are derived losfrom hematopoietic stem cells (HSCs) that reside in the bone marrow and migrate to the thymus. Most of the T cells generated in the thymus are short-lived, because they fail stringent rigorous selection criteria, which ensure that only cells bearing useful antigen receptors mature for export to the peripheral lymphoid tissues where they regulate immune responses. To survive,

immature thymocytes must express T-cell receptors (TCRs) capable of binding to molecules of the major histocompatibility complex (MHC) on thymic epithelial cells (positive selection). However, those with receptors which bind with high affinity to self-antigens presented by MHC molecules are induced to die by apoptosis (negative selection), thereby ensuring immunological tolerance (Strasser *et al.*, 1994) .

Unlike T cells, in mammals B cells stays in the bone marrow and do not migrate to another location to complete their maturation. The B cell contains a B cell receptor (BCR), which like the TCR is derived from the rearrangement of gene segments, is composed of two different proteins. In the case of the BCR, the proteins are the heavy chain (Hc) and light chain (Lc), which associate to form the bivalent four chain $(Hc)_2$ $(Lc)_2$ molecule.

The antigen specificity of BCR is identical to the antibodies, which will be secreted by its plasma cell descendants upon antigenic activation and differentiation. The surface Ig receptor has essentially no intracellular domain and so its signal transduction is through Hc-associated proteins Igα and Igβ. Like many receptors, crosslink of the BCR is the first step in the generation of a signal sufficient to begin a signalling cascade within the cell. B cells also contain complement receptors, which cross-linking with the BCR, resulting in an amplification of the signal (Harwood & Batista, 2010). Different of T cells, which can only recognize appropriately presented peptide antigens, B cells are able to recognize native antigen molecules binding to their BCRs. The B cell, however, require a secondary signal, which can be delivered through several pathways. The most common second signal is provided by the T cell and requires the interaction of a CD40L molecule on the surface of a T helper cell with the CD40 receptor on B cells. Some B cells can undergo T cell independent polyclonal activation. The independent provides a subset of B cells that recognize molecules on the surface of invaders not normally recognized by T cells, such as bacterial polysaccharides and lipids.

Whatever the stimulus, oncea B cell is activated further proliferation ensures but differentiate to secrete antibodies requires a cytokine signal. Upon activation, a B cell may alternatively to become a memory cell. Plasma cells, also called antibody-secreting cell secrete antibodies at a rate of 10.000 immunoglobulins per second for several days and then die (Slifka *et al.*, 1998). In contrast to plasma cells, memory cells retain their membrane-bound B cell receptors and continue to survive for longer periods of time, thus providing its classical secondary, or “memory” response, which is fundamental to vaccines. The secondary response to an antigen is faster, stronger, and has a higher affinity than the primary response. Memory B cells

are typically formed in immune responses that require T cell help. Although T cells are important to the immune response, they do not constitute a major component of the experiments performed in this thesis and, in contrast to B cells, they will not be discussed in further detail. The next section will describe, in greater detail, the process of B cell maturation.

1.5.1 B-Cell development and Differentiation

The differentiation pathway from stem cell to mature B lymphocyte can be divided into several stages characterized by proliferation, differentiation and apoptosis. This process starts with hematopoietic stem cells (HSCs) in the liver during fetal development and in the bone marrow (BM) after birth.

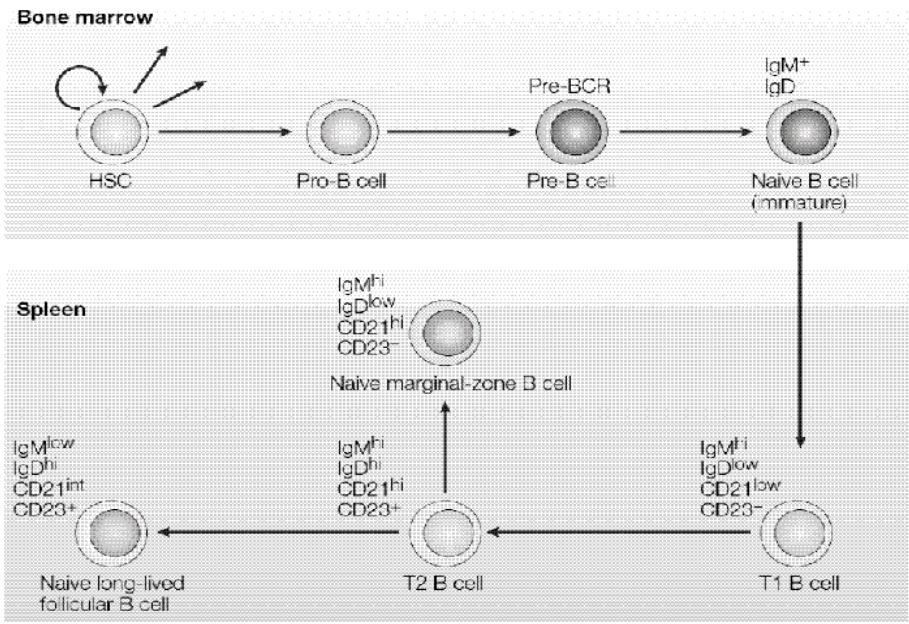

Figure 7. Antigen-independent development of B cells (Shapiro-Shelef & Calame, 2005).
B cells develop from pluripotent stem cells in the bone marrow, where full commitment to the B-cell lineage requires the transcription factor paired box protein 5 (PAX5). Naive B cells that exit the bone marrow continue to undergo maturation in the spleen to form long-lived naive follicular B cells and, to a lesser extent, naïve marginal-zone B cells.

In each sequential developmental stage, specific genetic programs are completed in specific environments. In general, cells either pass to the next step of differentiation or are eliminated by apoptosis.
In each sequential developmental stage, specific genetic programs Thus, in the early phases of differentiation in the bone marrow,

toproductively rearrange the H and L chain genes and the consequent signalling function of the BCR are essential requirements . The presence of the BCR is also indispensable for the survival of mature B cells in the periphery .The different stages of B-cell development can be identified by the presence of specific cell surface markers **(Figure 7)**. The earliest operationally B-lineage associated surface marker is a member of the common leukocyte antigen family CD45, and is usually referred as B220. It should be noted, however, that while some surface markers are lost after a stem cell becomes a precursor of a B cell, others, such as CD19, are gained. The development stage after a lymphoid progenitor is committed to become a B cell is the pro-B cell, which expresses the molecules B220 and CD43 on its surface. Following this, the pro-B cell differentiates into a pre-B cell, which expresses B220 on its surface, but does not express CD43, the pre-B cell then develops into an immature B cell, which has the surface markers B220 and IgM (Shapiro-Shelef & Calame, 2005). While all of the stages preceding the immature B cell stage are marked by a rearrangement of B cell receptor genes, the immature B cell possesses fully rearranged heavy/ light chain genes, which are expressed on the cell surface. Immature B cells further develop into mature naive B cells, which retain B220, IgM but not express IgD. Mature naive B cells are similar to effector B cells, but as they have not yet encountered antigen, they do not possess all of the characteristics of either plasma or memory cells. It is important to note that upon maturation, mature naïve B cells leave the bone marrow and enter the periphery, where they will be activated if they encounter an antigen for which they have specificity (Cambier *et al.*, 2007).

Upon activation, a B cell becomes a lymphoblast looses IgD and differentiates either into a plasma cell or a memory cell. Throughout the maturation process, diversity is generated between B cells as the B cell receptor molecule undergoes a series of changes due to the necessary integration of DNA sequences coding for their Variable J, D and constant region **(Figure 8)**.

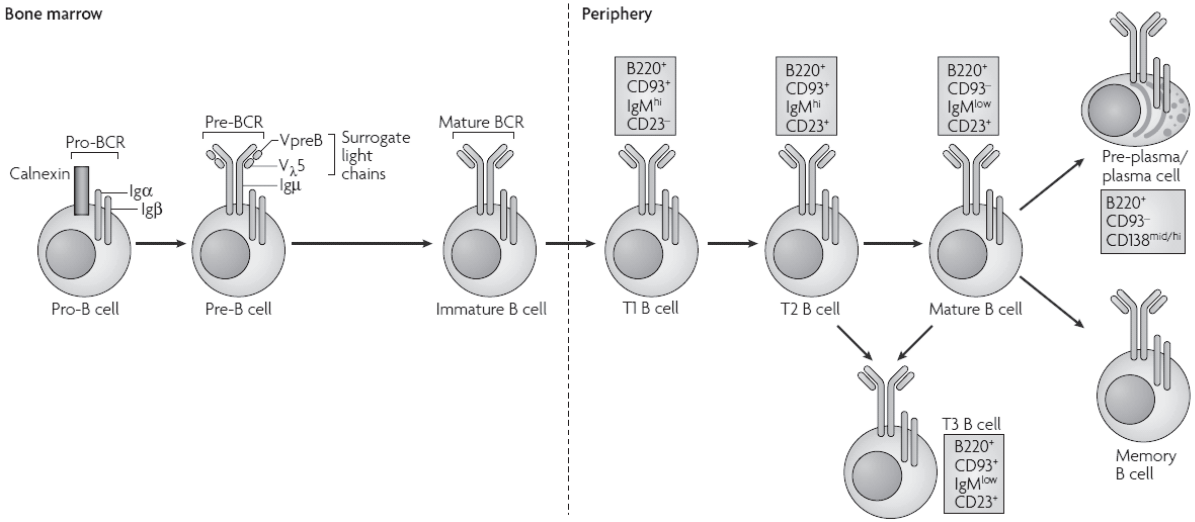

Figure 8. B-cell development occurs in both the bone marrow and peripheral lymphoid tissues such as the spleen (Cambier *et al.*, 2007).

In the bone marrow, development progresses through the pro-B-cell, pre-B-cell and immature-B-cell stages. During this differentiation, rearrangements at the immunoglobulin locus result in the generation and surface expression of the pre-B-cell receptor (pre-BCR, Igμ heavy chain and surrogate light chains V pre-B or Vλ5) and finally a mature BCR (rearranged heavy- and light-chain genes) that is capable of binding antigen. At this immature stage of development, B cells undergo a selection process to prevent any further development of self-reactive cells. Both receptor editing and clonal deletion have a role at this stage. Cells successfully completing this checkpoint leave the bone marrow as transitional B cells, eventually maturing into mature follicular B cells (or marginal-zone B cells). Following an immune response, antigen-specific B cells develop into either plasma (antibody-secreting) cells or memory B cells. Transitional 3 (T3) B cells, once thought to be part of the linear development from immature to mature B cells, are now thought to represent primarily self-reactive anergic B cells

When immature B cells are ready to exit the Bone Marrow (BM), they express on their surface CD19, intermediate levels of B220 and very low to high levels of IgM, the first Ig chain to be expressed. They do not express CD21, CD23 and only a small fraction expresses low levels of IgD (Carsetti *et al.*, 1995). The IgMhigh cells, enter the spleen through the terminal branches of central arterioles (Allman *et al.*, 1993; Lortan *et al.*, 1987). Only the B cells that express high levels of IgM and that have a functional BCR, leave the BM and enter the peripheral lymphoid system for subsequent development and differentiation into mature B cells (Rolink *et al.*, 2004). It is known that signalling through the BCR is essential for transition from the immature to mature B cells population to occur (Chung *et al.*, 2003). In fact, signalling through the BCR is essential in the development and survival of all B cells (Pillai *et al.*, 2004).

1.5.2 B-Cell immune function in host defence

By secreting antigen-specific immunoglobulin antibody, the B cell plays an essential role in host defence. Resting B cells reside in the circulation and migrate to the spleen and lymph nodes. Activation of the B-cell receptor by antigen, in conjunction with appropriate co-stimulation causes, the resting B cell to proliferate and differentiate into a plasma cell, which makes and secretes large amounts of immunoglobulin antibody. In addition to the antigen receptor, B cells exhibit a large number of additional cell surface receptors, which are activated by T-cell surface molecules, cytokines, bacterial endotoxin, and other ligands.

The key general steps in signalling pathways downstream of these receptors that regulate steps in development of the mature B cell and modulate responses to antigen are the following: First, antibodies can neutralize pathogens such as viruses and intracellular bacteria by binding to the cell surface and making the pathogen unable to enter the body's cells. Neutralization is also helpful in preventing bacterial toxins from entering cells. Second, antibody molecules can mark a pathogen for destruction by phagocytosis, a phenomenon called opsonisation. Third, antibodies can activate the complement protein system, causing the complement proteins to bind to the pathogen with the resultant opsonisation of the pathogen. Because the antibody response takes time to form, it is typically more critical during a secondary response than a primary response. While other mechanisms of immunity may be more effective in clearing the pathogen during a primary response, antibodies from differentiated memory B cells are extremely effective in stopping an infection upon the second introduction of a pathogen. Finally, B-cells are able to present specific antigens peptides to cognate CD4+ T cells with extremely high efficiency so that they obtain help for the production of high-affinity antibodies. The high-affinity BCR enables B cells to efficiently capture specific antigens present even at extremely low but immunologically relevant concentrations, far below those required for presentation by antigen-non specific B cells as well as DCs (Lanzavecchia, 1987; Pape *et al.*, 2007). Nonspecific antigens derived from endogenous self-peptide and pinocytosed proteins are also presented by B cells, but the outcome of presentation of non-specific antigen is T cell tolerance. Consequently, B cells can either activate or inactivate T cell, depending on the nature of the antigen. In the presence of dendritic cells or activated macrophages, the role of B cells in presenting nonspecific antigens is negligible.

Several studies have suggested a role for B cell in the induction of regulatory T cells (Mann *et al.*, 2007).

1.5.3 Molecular mechanisms underlying the B cell activation

B cell activation is followed by high affinity maturation in germinal centres (GCs), leading to the production of both plasma B cells capable of producing high-affinity antibodies and long-lived memory B cells (Rajewsky, 1996).Thus, the end of the adaptive immune response for the individual is the neutralization and elimination of antigen, accompanied by the acquisition of long-lasting protection from secondary challenge with the same pathogen.

B cell activation is initiated in response to specific antigen binding to the B cell receptor (BCR). The BCR is a complex comprised of membrane immunoglobulin (Ig) heavy and light chains, in association with the Igβ heterodimer (Reth, 1989). Engagement of BCR by antigen allows tyrosine phosphorylation of the intracellular Igβ immunoreceptor tyrosine activation motifs (ITAMs) by Src-family kinases, such as Lyn, leading in turn to the activation of Syk (Dal Porto *et al.*, 2004a). This activation initiates the coordinated assembly of the signalosome, composed of a variety of intracellular signalling molecules, such as Vav, Bruton's tyrosine kinase (Btk), (Dal Porto *et al.*, 2004b; Kurosaki, 1999) as well as adaptor proteins, such as B cell linker (Blnk) (Goitsuka *et al.*, 1998).

The collective accumulation and activity of signalling molecules within the signalosome triggers a variety of cellular processes, including regulation of gene expression, reorganization of the cytoskeleton, and BCR mediated internalization of antigen complexes. Antigen internalized through the BCR is subsequently processed inside specific endosomal compartments and presented in complex with MHC II to recruit specific T cell help (Lanzavecchia, 1985).

Hence, B cell activation is dependent on the organization of a range of molecules, requiring the synchronization of both intracellular signalling pathways and intercellular communication. Classic biochemical techniques have been used to gain valuable insight into the interactions of individual macromolecules, in response to B cell stimulation with model ligands, such as high affinity antibodies or soluble antigens.

1.5.4 The immunological synapse & B-Cell spreading

BCR signalling and cytoskeleton reorganization mediate B cell dendrite formation “spreading”, increasing the possibility of cell interactions and facilitating the formation of greater numbers of BCR clusters and thus contributing to cell activation. The immunological synapse, originally reported for CD4+ T cells (Grakoui *et al.*, 1999), is recognized as a common feature of antigen recognition by specific immune receptors (Batista *et al.*, 2001; Stinchcombe *et al.*, 2001).

The immunological synapse forms as a result of an extraordinary and coordinated reorganization of molecules in the membrane and cytoskeleton. The mature immunological synapse is characterized by an increase of immunoreceptor-antigen complexes in a central supramolecular activation cluster (cSMAC), surrounded by a ring of integrins forming the peripheral supramolecular activation cluster (pSMAC).

The immunological synapse plays an essential role in B cell activation. After recognition of membrane-bound antigen, the BCR signalosome is assembled by the highly coordinated and sequential recruitment of initiating kinases, such as Lyn and Syk; intracellular signalling molecules, such as Vav, PI3K, PLC2 and Btk; and adaptors, such as Blnk and CD19 (Harwood & Batista, 2010)

During B cell spreading, the contact area increases, allowing for the amplification of the generation of signalling BCR micro clusters and thus enhancing B cell activation. After spreading, a slower contraction occurs, which thought to be involved in the amplification of signalling BCR and formation of BCR micro clusters within the cSMAC, so that antigen can be internalized into endosomes for later antigen presentation to T helper cells.

The rapid spreading response, mediated by the extension of lamellipodial projections, allows B cells to engage more antigen as they progress over the antigen-containing surface and in that manner propagate signalling through the BCR. The degree of the spreading and contraction response is dependent again on the nature of the stimulating antigen, such that a higher antigen avidity stimulates spreading and B cell activation consequently (Fleire *et al.*, 2006). The resulting, more prolonged contraction phase then allows for the collection of more antigen into the central aggregate, which is subsequently internalized and presented to T cells, thus contributing to B cell activation (Lanzavecchia, 1985).

A number of intracellular signalling molecules involved in effecting the spreading response have been identified (Weber *et al.*, 2008). The absolute dependence of spreading on Src family kinases was seen by the abrogation of spreading in Lyn deficient B cells. After the

sequential recruitment and activation of Src and Syk kinases to BCR micro clusters, the cooperative action of PLC2 and Vav within these micro-signalosomes was also found to be critical for effective propagation of signalling to enable B cell spreading. A similar stabilization of signalling micro clusters through cooperation of their component parts had been previously noted in T cells (Bunnell *et al.*, 2001) and may favour a greater sensitivity discrimination between antigen and self-nonself. The correct spatiotemporal localization of these molecules through the activity of adaptor proteins such as Blnk is very important for the proper functioning of PLC2 and Vav in mediating B cell spreading (Weber *et al.*, 2008).

Considerable changes in B cell morphology during spreading and contraction. The identification of a key role for Vav in the propagation of the spreading response offers some insight into mechanisms underlying the regulation of cytoskeleton reorganisations. As such, Vav has been demonstrated to be involved in the deactivation of the ezrin-radixin-moesin (ERM) protein ezrin (Faure *et al.*, 2004), and, in addition, Vav can function as a guanine nucleotide exchange factor (GEF) for the cytoskeleton-modifying RhoGTPases (Jaffe & Hall, 2005). In line with this, it has been demonstrated that the Vav-dependent activation of Rac1 is required for BCR-mediated spreading in mature B cells (Brezski & Monroe, 2007). The activity of the small GTPase Rap, regulated by Vav, has been shown to perform a critical role in the activation of B cells through the regulation of spreading and immunological synapse formation (Arana *et al.*, 2008; Lin *et al.*, 2008). The resulting spreading response provides a mechanism for the propagation of BCR-mediated signalling and the facilitation of B cell activation.

The importance of Follicular Dendritic cells (FDCs) in the presentation of antigen to B cells has been well established. Some studies based in dynamic visualization of GC B cells after administration of antigen suggested that B cells may not occupy a fixed position on the surface of FDCs (Allen *et al.*, 2007; Schwickert *et al.*, 2007). However, Hauser *et al.* were able to identify a population of relatively stationary B cells, after antigenic stimulation, forming close associations with FDCs. Then, B cells can encounter antigen through a variety of different mechanisms *in vivo* (Harwood & Batista, 2008).

The mechanism for each particular antigen is dependent both on the properties of the antigen itself and its route of entry into the body. The studies observe that B cells within the GC adopt an unusual, heterogeneous morphology with many elongated processes. In the light of these investigations, it has been proposed that even migrating B cells may be capable of sequestering antigen through their

extended processes as they crawl across the surface of FDCs (Allen *et al.*, 2007). This antigen could subsequently be presented to T helper cells, suggesting a possible mechanism whereby the highest affinity B cell clone would be the most able to compete effectively for specific T cell help.

1.5.5 Germinal Center

Germinal centres develop in the B cell follicles of secondary lymphoid tissues during T cell-dependent (TD) antibody responses. The B cells that give rise to germinal centres are initially activated outside follicles in the T cell-rich zones through T cell help. The germinal centres formed are oligoclonal and results from massive clonal expansion (MacLennan, 1994). During this step, a site-directed hypermutation mechanism is activated that acts on their immunoglobulin-variable (Ig-v)-region genes. At the end of the period of exponential growth of B blasts, the classical structure of germinal centres emerges. The B blasts become centroblasts in the dark zone of the germinal centres, which develops at that limit of the FDC-network close to the T zones. The centroblasts are still in rapid cell cycle but maintain a stable sized population as their progeny move to the heart of the FDC-network where they exit cell cycle as centrocytes (Allen *et al*., 2007; Shlomchik & Weisel, 2012). Centrocytes seem to be selected for their ability to interact with antigen bound to FDCs. There is a high death rate among centrocytes in vivo, and when these cells are isolated *in vitro*, they undergo apoptosis within hours of culture. The long-term survival is achieved by signalling through their surface CD40. After activation through CD40, centrocytes increase their surface Ig and acquire characteristics of memory cells and recognition of antigen presented by FDC and its presentation to T cells, which can be induced at the point of cognate interaction. Centrocytes positively selected through interaction with antigen on FDC receive further signals, which induce the cells to differentiate to become either plasma cells and express CD40 ligand or memory B cells. The affinity maturation of antibodies that occurs during immune responses to protein antigens involves site-directed hypermutation of immunoglobulin variable-region genes that is activated in germinal centres (MacLennan, 1994; Victora & Nussenzweig, 2012) **(Figure 9).**

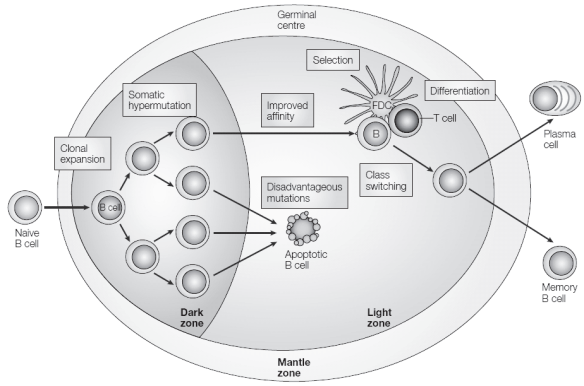

Figure 9. Mature germinal centres are divided into dark and light zones (Küppers, 2003).
The proliferating blasts, centroblasts, occupy the dark zone and give rise to centrocytes that are not in cell cycle and fill the light zone. The light zone contains a rich network of follicular dendritic cells (FDC) that have the capacity to take up antigen and hold this on their surface.

Germinal centres persist for about 3 weeks following immunization, but after this, memory B blasts continue to proliferate in follicles throughout the months of T cell-dependent antibody responses. These cells are probably the source of memory cells.

1.5.6 Immature and mature B-cell subpopulations in the spleen

Most of the successfully generated immature splenic B cell population will reach maturity, and data from several authors indicates that at this stage of transition from immature to mature B cells, negative selection will no longer take place (Coutinho *et al.*, 1995; Rolink *et al.*, 1998)
All immature splenic B cells can be subdivided according to their surface expression of IgM, IgD, CD21, CD23 and CD1d, and the current classification of B cell subpopulations is based on the differential expression of these markers. Immature splenic B cells express CD93, which distinguishes from the mature cells that do not express this marker **(Figure 7)** (Rolink *et al.*, 1998)
The immature subpopulations of B cells in the spleen can be divided into the newly formed (NF) or Transitional 1 (T1) cells. The T1 cells are characterized by high and low expression of IgM and IgD, respectively, and by the absence of CD21 and CD23. In the more mature Transitional 2 (T2) population, expression of IgM, IgD, CD21 and CD23 is high. Also, the T2 subpopulation shows lower levels of CD93, compared to the T1 population (Cambier *et al.*, 2007; Dal Porto *et al.*, 2004b; Rolink *et al.*, 2004). The Transitional 3 (T3) subpopulation is characterized by the surface expression of high levels of IgD, CD1d,

CD21 and CD23, and intermediate levels of IgM **(Figure 7)** (Rolink *et al.*, 2004; Shapiro-Shelef & Calame, 2005). In mice, the ratio between the T1:T2:T3 subpopulation is 1:1:1 and is not altered with the age of the animal, but as the mice get older, there is a decline in the proportional contribution of immature B cells to the pool of total splenic B cells (Rolink *et al.*, 2004).

As the immature B cells enter the lymphoid follicles of the peripheral lymphoid system, they acquire the more mature follicular phenotype (FO). These cells express high levels of IgD and CD23, intermediate levels of CD21, low levels of IgM, and progressively lower levels of CD93 and CD24 (Rolink *et al.*, 1998, 2004). This follicular population is the main cell type in the pool of naive B cells in the spleen, with relatively short half-life of around, 2-3 months (Pillai *et al.*, 2004). Some FO B cells will migrate to the marginal sinuses in the spleen and differentiate into marginal (MZ) zone B cells. These are larger than FO B cells, have characteristically and distinctively crinkled membranes (Oliver *et al.*, 1999) and are mostly located between two concentric layers of macrophages (Pillai *et al.*, 2005). Marginal zone B cells are $IgM^{high}IgD^{low}CD21^{high}CD1d^{high}CD23^{-}$, and do not differentiate to plasma cells in response to specific antigen receptor ligation. They are, however, quickly activated, expressing high levels of CD80 (B7-1) and CD86 (B7-2), and stimulation with lipopolysaccharide (LPS), anti-CD40 and IL-4 may result in their differentiation into plasma cells within a matter of hours (Oliver *et al.*, 1999). Their proliferation in response to LPS is much more vigorous than that of FO B cells and they will fail to develop in the absence of CD19 (Pillai *et al.*, 2005).

It is accepted that FO B cells are important for the T-dependent germinal centre-based responses, while MZ B cells seem to contribute to T-independent responses to blood-borne pathogens, even though they may also contribute to T-dependent responses, because, since they express high levels of CD80 and CD86, they might present blood-borne pathogens to naïve T cells (Pillai *et al.*, 2004).

1.5.7 B1 cells: development and selection

The peritoneal cavity of mice is enriched for B-1 B cells, a lymphocyte subset that differs phenotypically, functionally, and developmentally from the conventional B-2 B cells. This long-lived, self-renewing subset of cells is predominantly abundant in young individuals and, in the mouse, is present mainly in the peritoneal cavity, with small numbers also detectable in the spleen and lymph nodes of adult mice (Pillai *et al.* 2004). They are especially involved in T-independent responses to antigens present in the intestinal mucosa. In normal mice, B-1 B cells produce a significant proportion of natural IgM

autoantibodies in the serum, and display an enriched expression of self-reactive BCRs (Hardy, 2006; Pillai *et al.*, 2004).

These B-1 cells are $IgM^{hi}IgD^{-}CD23^{-}CD11b^{+}$ and can be further subdivided in the B-1a and B-1b, with the distinguishing characteristic of CD5 surface expression by B-1a but not B-1b subpopulations (Hardy *et al.*, 1996; Herzenberg *et al.*, 1986) (Hardy and Hayakawa 1986; Herzenberg *et al.* 1986; Hardy and Hayakawa 1992). The B-1a cells develop from HSCs derived from the foetal liver (Hayakawa *et al.* 1985; Hardy and Hayakawa 1992), while B-1b originate from HSCs from both foetal liver and BM (Hardy & Hayakawa, 1994; Kantor *et al.*, 1992). Proliferation of both B-1 B and B-1a B cells generation requires BCR signalling. In fact, all the components of the Btk/PLC-/PKC-pathway are necessary for the generation of B1 B cells, but it seems that B1b B cells can sometimes be generated in bacterially infected Xid mice (Pillai *et al.*, 2004). Work from several groups has demonstrated that B1-b cells are critically and more strongly dependent than B2 B cells on normal BCR signalling for their development and/or maintenance (Hardy, 2006).

1.5.8 T cell dependent and T cell independent activation

In order for B cells to carry out their effector functions they must first be activated by encountering the pathogen for which they are specific. Antigen recognition is not the only necessary event for activation, however. In most cases, B cells must also receive a signal from T cells, and in other cases, they must receive a special kind of signal from the pathogen. These two types of responses are termed thymus-dependent (TD) and thymus-independent (TI) humoral responses **(Figure 10)**. While the TD response is more typical, TI responses are also important for defence of against bacterial pathogens. In the thymus-dependent response, an antibody response by B cells requires help from T cells, which recognize the same antigen.

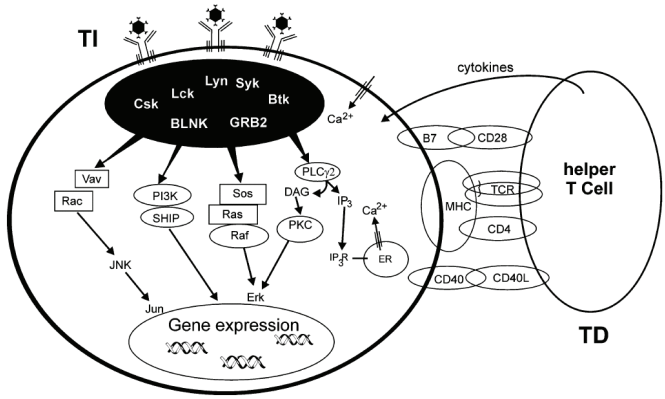

Figure 10. B cell activation by T cell-dependent and T cell-independent antigens (Ollila & Vihinen, 2005).
A large number of similar antigens can directly initiate B cell activation (TI, top and TD, right). T cell-dependent antigens are degraded into small peptides and presented to T cells by MHC molecules. T cell receptors recognize the antigen and B cells become activated. Many other cell surface proteins are also important for this event. Several signalling pathways are activated after BCR stimulation as well as the kinases and adaptor proteins involved

This critical interaction of a B cell and T cell specific for the same antigen is termed linked recognition. The B cell and T cell do not need recognize the same epitope on the antigen; they must only recognize epitopes on the same antigen molecule. In order for a B cell to be fully activated, it must receive help from an activated T helper (Th) cell. A T-helper cell can be activated by any antigen-presenting cell (dendritic, macrophage or B cells) displaying its cognate antigen presented in the context of MHC class-II molecule. Upon recognizing an antigen, a B cell will travel to the border between the T cell zone and B cell zone of

the lymph node, where activation occurs if it encounters a T cell with specificity for an antigenic peptide presented by class I MHC. CD40 on the B cell also binds to its ligand, CD40L (also called CD154), on the T cell. The activation also involves the interaction with cytokines such as IL-4 and IL-5. Activated B cells then begins to proliferate and differentiates, forming a germinal centre. From the germinal centre, cells are released that become plasma cells, travelling through the periphery to the bone marrow and spleen to secrete antibody molecules. In the model of a TD humoral response, helper T cells are necessary to create an effective antibody response to a pathogen

Although the thymus-dependent response is the typical mechanism of humoral response, in some cases B cells do not require the help of T cells in order to become activated. These thymus independent antibody responses are directed to responses and are divided into those against antigens that are also mitogen such as LPS, CpG, or poly-IC, (TI-I response) and those against antigens that have no intrinsic mitogenic activity and composed of highly repetitive structures, such as bacteria capsular polysaccharides, Dextran or Ficoll (TI-II response) (Obukhanych & Nussenzweig, 2006) .

TI-II antigens are typically repetitive polysaccharides of bacterial surfaces, which activate B cells through an extensive BCR-crosslinking. B cells respond to TI-II antigens in the absence of MHC class II-restricted T-cell help by a mechanism that depends on the expression of a functional Bruton's tyrosine kinase (Btk). These responses are lost or absent in neonates and in hosts with congenital or reduced defects in the expression of a functional Bruton's tyrosine kinase and distinguish them from the T-cell-independent type 1 (TI-I) antigens (Mosier *et al.*, 1977).

The TI-II response is critically dependent on the formation of a small number of antigen receptor clusters that induces a local membrane association of multiple activated Btk molecules, which results in long-term mobilization of intracellular ionized calcium. This persistent calcium fluxes efficiently recruit transcription factors and induce T-cell-independent B-cell activation and proliferation. There are studies suggesting that while this first signal of multivalent membrane Ig cross-linking can induce B-cell proliferation, TLRs could recognize molecular motifs on the surface of pathogens and provide the TI-2-activated B cell with a second signal (Coutinho *et al.*, 1974; Vos *et al.*, 2000).

For this type of antigen at concentrations that are too low, there is insufficient cross-linking of the BCR to activate the B cell and at high concentrations, the cell enters in apoptosis. While TI antigens are efficient at activating B cells, and may do so more quickly than TD antigens because of the lack of requirement for T cells, they are largely

inefficient at generating B cell memory. Their principal function is to provide a rapid primary response to microbial infections (Defrance *et al.*, 2011).

1.6 Murine Herpes Virus (MHV-68)

1.6.1 Pathogenicity of MHV-68

Gammaherpesviruses are lymphotropic viruses which establish lifelong infections in their hosts and are associated with cellular transformation and the development of malignancies, particularly in immunosuppressed individuals (Nash *et al.*, 2001). The MHV-68 virion exhibits morphological similarity to the virion organization of other gammaherpesviruses. The viral genome encodes canonical capsid, tegument, and glycoprotein homologues found in gammaherpesviruses (Virgin *et al.*, 1997).

The overall similarities of MHV-68 to other gammaherpesviruses in terms of genomic organization, pathological symptoms and the establishment of both lytic and latent infection make it a valuable animal model for human herpes viruses. Closely related to EBV and KSHV (Clambey *et al.*, 2002) this small-animal model can give clues to the versions of human gammaherpesviruses.

By analogy with others animal gammaherpesviruses MHV-68 may naturally be transmitted by a mucosal route of infection where the respiratory tract is likely to be a primary target. Although the experimental infection via the intra-peritoneal route does not represent the physiological situation, this approach has merit for evaluating the pathogenesis of replication-defective mutant viruses. At this time, the natural route of transmission is unknown and there are some documented cases of MHV transmission between animals infected when a mother ate her infected pups (Blaskovic *et al.*, 1984), or transmission via milk of infected mothers (Raslova *et al.*, 2001).

1.6.2 Immunity against MHV-68

Following intranasal inoculation of mice with MHV-68, a productive infection occurs in the lung. This is cleared around day 10-post infection (p.i.), principally by CD8+ T cells. At about this time, the virus spreads to the spleen, where it becomes latent mostly in B lymphocytes, macrophages and dendritic cells, but later predominantly in B lymphocytes (Dutia *et al.*, 1999; Simas & Efstathiou, 1998). The establishment of the latent state is first characterized by a transient

expansion of infected germinal centre (GC) B cells in splenic follicles, followed by long-term latent infection, probably in memory B cells. As has been proposed for Epstein Barr virus (EBV), the infection of GC B cells by MHV-68 may constitute a strategy to expand the latently infected cell pool and gain access to long-lived memory B cells (Nash *et al.*, 2001).

1.6.3 Gamma-herpes virus proteins involved in host immune evasion

Herpes viruses have double-stranded DNA genomes of 100 to 250kbp, consisting of up to 225 open reading frames (ORFs). These large and complex genomes can code for a huge repertoire of gene functions evolved for manipulation of host cell biology and immune responses. Although the complete sequence of the genome of many herpes viruses has been determined, the precise function for many of the viral genes, both *in vitro*, and in particular *in vivo*, has not been elucidated (Nash *et al.*, 2001).

In summary, gamma-herpes viruses behave like many other persistent viruses. They exploit naive host to replicate extensively in an exaggerated primary infection and establish a sizeable pool of latent genomes. Latency then provides a relatively stable, immunologically silent mode of persistence. Reactivation from latency allows virions to be shed and transmitted to new hosts, where the cycle begins again. Gamma-herpes viruses achieve a long-term infectivity order of magnitude higher than pathogens such as HIV and hepatitis C virus. This high efficiency of exit from the persistently infected host has made them ubiquitous pathogens. Host immunity generally prevents disease, but not viral transmission, most probably because immune evasion keeps low level reactivation below the threshold of immune recognition (Stevenson & Efstathiou, 2005). The same immune evasion makes EBV among the first pathogens to cause disease when there is immune suppression.

B cells are very important for systemic viral dissemination and probably for transit back to mucosal surfaces, where there is reactivation and viral shedding. B cells may also traffic virus to and from non-lymphoid sites of latency such as vascular endothelium (Weck *et al.*, 1997). ORF72 and ORF74 contribute to efficient *ex vivo* reactivation, but whether they are also important for *in vivo* reactivation remains unproven. Gp150 is required for efficient viral release from infected cells and is therefore likely to be required for virion shedding. MHV-68 also encode,

mK3 protein, that downregulate MHC-I (Stevenson *et al.*, 2000) and a viral bcl-2 homolog, a viral cyclin, and a chemokine-binding protein, M3 (Van Berkel *et al.*, 1999; van Dyk *et al.*, 1999; Gangappa *et al.*, 2002).

The left end of the unique region of the MHV-68 genome has attracted considerable interest due to the presence of four open reading frames (ORFs) (M1, M2, M3, and M4) and eight viral tRNA-like genes (vtRNAs), none of which have a homologue in viral or eukaryotic proteins, suggesting that these genes may perform a unique function for the virus as they share positional homology to latency-associated genes in other gammaherpesviruses.

To understand the mechanisms by which gammaherpesviruses maintain a latent infection while evading destruction by the host immune response, research has focused on genes transcribed during latency.

M2 ORF has attracted particular interest because its expression is restricted to latent infection *in vitro* and *in vivo*, with a peak of expression the spleen around day 14 p.i. M2 is expressed predominantly, if not solely, within B cells and not in splenic macrophages and dendritic cells that also harbour latent virus (Macrae *et al.*, 2003). Moreover previous reports based in results in B-cell-deficient mice point out the possibility that M2 expression in the spleen was not exclusive to B cells as M2 was detected in peritoneal exudates cells (PECs) following intraperitoneal inoculation of μMT knockout mice which fail to develop mature B cells (Virgin *et al.*, 1999), indicating that macrophages could also be a reservoir for latent MHV-68 (Weck *et al.*, 1999). Although these reports would seem to be inconsistent with the hypothesis that M2 expression is B-cell-restricted, an alternative explanation is that μMT mice do indeed generate B cells, through an μM-independent pathway that specifically generates immunoglobulin A (Macpherson *et al.*, 2001).

Analysis of the sub localization of M2 protein in A20 B cell line demonstrated a cytoplasmic and plasma membrane associated localization (Macrae *et al.*, 2003). Its localization suggests a possible role in cell signalling, also suggested by several studies *in vivo*. M2-deficient MHV-68, which is deficient in latency amplification, has more latency than wild-type virus late in infection, predominantly in germinal centre B cells (Simas *et al.*, 2004).

1.6.4 Function of the M2 protein

The M2 open reading frame (ORF) is located in a cluster of unique genes at the left end of the MHV-68 genome and shares positional homology with latency and transformation-associated genes present in other characterized gammaherpesviruses (Virgin & Speck, 1999). The M2 gene was shown to be transcribed in latently infected tissue *in vitro* and to encode a latency-associated antigen in the MHV-68 latently infected murine B-lymphoma cell line S11 (Husain *et al.*, 1999). The gene encoding this protein is located near the 5' end of the MHV-68 genome. Initial analysis of the M2 transcript revealed a spliced transcript composed of two exons, i.e., a noncoding 110-nucleotide (nt) 5' exon and a 1,235-nt 3' exon that contains the M2 ORF, and a 656-nt 3' untranslated region (Husain *et al.*, 1999). Analysis of M2 gene transcription in latently infected mice identified a novel transcript arising from the region of the M2 gene, which contains a short ORF (M2b). The analysis of viruses lacking an intact M2b failed to identify a role for this putative gene product in MHV-68 infection (DeZalia & Speck, 2008). However, the organization of candidate cis elements identified upstream of the distal promoter was consistent with the hypothesis that this promoter may be differentially utilized suggesting the possibility that distinct transcription regulation of the M2 gene may be involved in the role of the M2 protein in establishment of latency versus its role in reactivation from latency (DeZalia & Speck, 2008).

An examination of its primary sequence indicates the presence of multiple proline-rich regions (PRRs) and potential phosphorylation sites. Although M2 is a unique protein with no known homologues in viruses, mice, or humans, inspection of the amino acid sequence reveals many hallmarks of a bona fide signalling molecule, including 9 PXXP motifs and a central positively charged region, leading to the hypothesis that M2 may manipulate signal transduction pathways. Biochemical analysis suggested that M2 is capable of interacting with a number of SH3 domain-containing proteins, including Vav1, Vav-2, and Vav-3; Fyn; the tyrosine kinases TXK and Tec; Nck2; Grb2; endophilins II and III; Ras GTPase-activating protein 1; and Rho GTPase-activating protein 4 (Madureira *et al.*, 2005; Rodrigues *et al.*, 2006). In fact, specific key SH3 binding motifs and tyrosine phosphorylation motifs contribute significantly to the establishment of latency and reactivation *in vitro* (Herskowitz *et al.*, 2008). Furthermore, it was demonstrated in B-cell lines by others that three PXXP motifs located in the c-terminal half of M2 are required to bind Vav1 and induce phosphorylation of Vav1 leading to downstream Rac1 stimulation (Madureira *et al.*, 2005;

Rodrigues *et al.*, 2006). Additionally, M2 activates the Vav1/Rac1 pathway through a trimolecular complex with Vav1 and Fyn, leading to enhanced Vav1 phosphorylation (Pires de Miranda *et al.*, 2008; Rodrigues *et al.*, 2006). Vav proteins are enzymes that promote GDP/GTP exchange on Rho/Rac proteins in a phosphorylation-dependent manner, thereby favouring the rapid transition of those GTPases from their inactive (GDP-bound) to active (GTP-bound) states during signal transduction. The activation of Rho/Rac proteins promotes extensive changes in intracellular pathways related to cytoskeletal change, mitogenesis, and cell survival (Rodrigues *et al.*, 2006).

Others *in vitro* studies have demonstrated that the M2 protein has a cell-type-dependent localization and effectively inhibits interferon-mediated signal transduction by downregulation of STAT1/2 expression (Liang *et al.*, 2004) and inhibits apoptosis induced by DNA damage via interaction with the DDB1/COP9/cullin repair complex and the ATM DNA damage signal transducer respectively (Liang *et al.*, 2006) similar to the impact of Kaposi's sarcoma-associated herpesvirus (KSHV) infection (Shin *et al.*, 2006). However, these findings were not confirmed by *in vivo* experiments using MHV-68 M2 deletion mutant viruses.

M2 expression in primary B cells leads to interleukin-10-dependent B-cell proliferation and secretion of IL-10, IL-2, MIP-1a, and IL-6 (Siegel *et al.*, 2008). Primary B cells expressing M2 differentiate into a pre-plasma memory B-cell phenotype, an intermediate differentiation state between plasma and memory B cells (Siegel *et al.*, 2008).

1.6.5 M2 gene in the virus-host interaction

In order to establish the role of M2 gene in the virus-host interaction various studies were done using different M2-deficient MHV-68 strains to evaluate their pathogenesis. The first mutant made by Jacoby *et al.*, 2002 was made by introduction of a stop codon and an elevation of the level of genome-positive cells in the spleen after peritoneal infection was also observed. M2 was differentially required for acute replication *in vivo*. While mutation of M2 did not affect acute phase of virus replication in the lungs of mice following intranasal inoculation, acute-phase virus replication in the spleen was decreased compared to that of the wild-type and marker rescue viruses following intraperitoneal inoculation.

Upon intranasal inoculation, M2 mutant viruses exhibited a significant decrease in the establishment of latency in the spleen on day 16-post infection, as measured by the frequency of viral genome-positive cells. In addition, M2 mutant viral genome-positive cells reactivated from

latency inefficiently compared to wild-type and marker rescue viruses. By day 42 after intranasal inoculations, the frequencies of M2 mutant and wild-type viral genome-positive cells were nearly equivalent, and little reactivation was detected from either population. In sharp contrast to the results obtained following intra nasal inoculation, after intraperitoneal inoculation, no significant defect was observed in the establishment or reactivation from latency with the M2 mutant viruses indicated that the requirements for the establishment of latency are affected by the route of infection (Clambey *et al.*, 2002).

The second mutant performed by Macrae *et al.*, 2003 was made by a frameshift mutation in the M2 open reading frame that caused premature termination of translation of M2 after amino acid residue 90. The M2 mutant showed no defect in productive replication *in vivo* or in lungs after infection of mice. Likewise, the characteristic transient increase in spleen cell number, Vbeta4 T-cell-receptor-positive CD8+ T cell mononucleosis, and establishment of latency were unaffected. However, the M2 mutant virus was defective in its ability to cause the transient sharp rise in latently infected cells normally seen in the spleen after infection of mice. This study also reported that the expression of M2 is restricted to B cells in the spleen and that M2 encodes a 30-kDa protein localizing predominantly in the cytoplasm and plasma membrane of B cells.

Given that M2 was required for the expansion of latently infected cells within the spleen during acute infection, it was somewhat surprising that mutation of M2 did not negatively affect the establishment of long-term latency. This is supported by similar observations obtained with other M2 mutants (Jacoby *et al.*, 2002) and with MHV-76 (Macrae *et al.*, 2001). However, other experiments with M2 deficient MHV-68 viruses demonstrated that the absence of M2 results in reduced levels of splenic latency at short times after infection. This deficit was correlated with a decreased number of infected follicles rather than inability to expand the latent load in GC B cells. In spite of this, a lack of M2 was associated with increased long-term levels of latency in germinal centre B cells. This phenotype has been correlated with a deficit of latency establishment in memory B cells (Simas *et al.*, 2004). Additionally, infection with an M2 null MHV-68 (M2.Stop) leads to a significant decrease in serum IL-10 levels at day 16-post infection, correlating with an increase in the frequency of MHV-68-specific CD8 T cells (Siegel *et al.*, 2008). Mechanism that is consistent with previous analyses of MHV-68 mutants that have suggested a role for the M2 protein in MHV-68 pathogenesis promoting the expansion and

differentiation of MHV-68 latently infected B cells (perhaps facilitating the establishment of virus latency in memory B cells).
It was also reported that the 193-amino-acid M2 protein contains an immunodominant CD8+ T-cell epitope, suggesting that immune recognition of M2-expressing cells is critical for the resolution of splenomegaly and, perhaps, the maintenance of the host-virus equilibrium that underlies viral persistence (Husain *et al.*, 1999).

1.6.6 MHV-68 reactivation

Chronic gamma-herpesvirus infection is a dynamic process involving latent infection, reactivation from latency, and low-level persistent replication. The gamma-herpes viruses maintain latent infection as a result of an complex balance between host factors that suppress infection and viral factors that facilitate evasion of the immune response. Immune effectors limit reactivation and subsequent replication events, and the adaptive immune response ultimately restricts infection to a level compatible with life-long infection (Tibbets *et al.*, 2006). Both innate and adaptive immune responses play an active role in limiting virus infection. The absence of type I interferon (IFN) results in a lethal MHV-68 infection, emphasizing the central role of these cytokines at the early stages of infection. In contrast, type II IFN is not essential for the recovery from infection in the lung,but a failure of type II IFN receptor signalling results in the atrophy of lymphoid tissue associated with virus persistence (Barton *et al*., 2011).

CD8+ T cells play a major role in recovery from the primary infection, and also in regulating latently infected cells expressing the M2 gene product. CD4+ T cells have a key role in surveillance against virus recurrences in the lung, in part mediated through the collaboration on the generation of neutralizing antibodies. In the absence of CD4+ T cells, virus-specific CD8+ T cells are able to control the primary infection in the respiratory tract, yet surprisingly the memory CD8+ T cells generated are unable to inhibit virus recurrences in the lung (Nash *et al.*, 2001).This could be explained in part by the observations that these viruses can downregulate MHC-I expression and also restrict inflammatory cell responses by producing a chemokine-binding protein (M3 gene product) (Smith *et al.,* 2007).
The maintenance of chronic MHV-68 infection, at least in some latency reservoirs, appears to be dependent on the capacity of the virus to reactivate from latency *in vitro*. However, the signals that lead to MHV-68 reactivation *in vivo* are not well characterized. Stimulation of latently infected B cells appears to be a critical component

to the reactivation of gamma-herpes viruses. This hypothesis has been supported by the inhibition of EBV lytic replication of anti-Ig stimulated cells using the immunosuppressive drugs cyclosporine A and FK506 (Durandy, 2001). It was reported that the reactivation of MHV-68 from latently infected splenic B cells at late times post-infection can similarly be triggered by either anti-Ig/anti-CD40 or LPS stimulation (Moser *et al.*, 2005). This studies draw attention to the idea that there were common intracellular signalling pathways that, in general, lead to gammaherpesvirus reactivation from latently infected B cells. Later, it was described that stimulation of latently infected B cell lines with ligands for TLRs 3, 4, 5, and 9 enhanced MHV-68 reactivation; the ex-vivo stimulation of latently infected primary splenocytes, recovered from infected mice, with poly (I:C), LPS, flagellin, or CpG DNA led to early B-cell activation, B-cell proliferation, and a significant increase in the frequency of latently infected cells reactivating the virus (Gargano *et al.*, 2009). And, interestingly, *in vivo* TLR stimulation also induced B-cell activation and MHV-68 reactivation, resulting in higher levels of virus replication in the lungs which correlated with an increase in MHV-68 specific CD8+ T-cell responses, demonstrating that TLR stimulation can drive MHV-68 reactivation from latency and suggests that periodic pathogen exposure may contribute to the homeostatic maintenance of chronic gammaherpesvirus infection through stimulating virus reactivation and reseeding latency reservoirs (Gargano *et al.*, 2009).

1.7 Scope and outline of this Thesis

The advent of the genomic era has brought a huge amount of viral genome information. Biological databases have flourished in last years and the development of tools capable of analysing this information has provided a valuable tool for rational approaches to the study of the function of viral genes. At the same time, the progressive advances in the molecular biology have permitted the design of innovative approaches to dissect the precise roles of viral proteins. Thus, during the past decades, the identification of the key molecular properties and signalling pathways of viral genes have contributed much to our understanding of the pathogenesis of many viruses.

The work of this thesis focuses on an analysis of two virus host evasion genes in two very different circumstances, the *in vitro* and the *in vivo*, with the shared understand their contribution to the complex dialogue between the pathogen and host.

The first gene, ORF I329L, from African Swine Fever (ASFV) was identified and characterized following the hypothesis that this virus, as a pathogen of both ticks and pigs, would have evolved genes to

manipulate the innate immune response, as it is a common denominator between vertebrate and invertebrate animals. It was selected on the basis of a very marginal sequence homology with Toll-like receptors, the subsequent confirmed by functional studies on the impact of the virus gene *in vitro*.

The second gene, M2 of MHV-68, was selected because of its known association with the establishment of latency in B cells, and the strategy, accordingly, was to generate B-cell restricted transgenic expression of M2 and then to restricted transgenic expression of M2 and then to assess the impact of the transgene on the ontogeny and function of B-cells *in vivo*, the latter in response to function of B-cells *in vivo*, the latter in response to conventional antigens and infections with wild-type and M2 deficient MHV-68 viruses.

The work developed to achieve these goals is presented in this dissertation in three chapters:

Chapter 2 - A novel TLR3 inhibitor encoded by African Swine Fever Virus (ASFV)

Chapter 3 - The impact of B cell restricted transgenic expression of a Mouse Herpes Virus (MHV-68) host evasion gene

Chapter 4 - Evaluation of the M2 role in the MHV-68 Pathogenesis through an innovative approach.

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02 | A Novel TLR3 Inhibitor Encoded by African Swine Fever Virus (ASFV)

1 Abstract

African swine fever virus (ASFV) encodes proteins that manipulate important host antiviral mechanisms. Bioinformatic analysis of the ASFV genome revealed ORF I329L, a gene without any previous functional characterization as a possible inhibitor of TLR signaling. We demonstrate that ORF I329L encodes a highly glycosylated protein expressed in the cell membrane and on its surface. I329L also inhibited dsRNA-stimulated activation of NFkB and IRF3, two key players in innate immunity. Consistent with this, expression of I329L protein also inhibited the activation of interferon-β and CCL5. Finally, overexpression of TRIF reversed I329L-mediated inhibition of both NFkB and IRF3 activation. Our results suggest that TRIF, a key MyD88-independent adaptor molecule, is a possible target of this viral host immunomodulation gene. The demonstration of an ASFV host evasion molecule inhibiting TLR responses is consistent with the ability of this virus to infect vertebrate and invertebrate hosts, both of which deploy innate immunity controlled by conserved TLR systems.

2 Introduction

African swine fever virus (ASFV) is a lethal haemorrhagic pathogen of domestic pigs. In its natural mammalian hosts, the Warthog and Bushpig, however, the virus is persistent, non-pathogenic and transmitted by an invertebrate vector, the soft tick. The virus may therefore be considered as a veterinary model for recently emerged haemorrhagic infections. AFSV is an enveloped DNA virus with icosahedral morphology that is now classified as the only member of a new virus family, Asfarviridae (Dixon *et al.*, 2004). It exhibits a characteristic genomic structure, large numbers of novel genes and a cytoplasmic replication strategy, and, in contrast to all other DNA viruses, this arbovirus infects both vertebrate (swine) and invertebrate (tick) hosts. The ability of the virus to infect macrophages and to persist in its natural hosts, and in domestic pigs, which recover from infection with less virulent isolates, shows that the virus has effective mechanisms to evade host defense systems, in particular, innate immunity. It is estimated that half to two-thirds of the approximately 150 genes encoded by ASFV do not have known viral or cellular homologs (Chapman *et al.*, 2008). Most of the structural proteins and enzymes have been identified, but many of these non-homologous genes have no homology to known proteins or enzymes. Therefore, we hypothesize that some of these genes have evolved for, or may be involved in, host evasion. Such genes provide a source of potentially valuable tools for understanding virus pathogenesis and for identifying novel aspects of the vertebrate immune system. The fact that ASFV has adapted to infect both mammalian macrophages and an invertebrate tick host suggests that this virus may have evolved immune evasion genes focused on innate immunity, more specifically, for manipulation of the Toll-like receptor (TLR) signalling system, which is conserved in both mammals and arthropods. Moreover, there is abundant expression of TLRs in macrophages, where they play a critical role in the detection of viral infections, leading to the inflammatory response and induction of an interferon (IFN)-mediated anti-viral state in infected and neighbouring cells (Kawai & Akira, 2007). Under some circumstances, however, uncontrolled activation of macrophages leads to undesirable pathological consequences.

To date there are at least 13 distinct mammalian TLRs, all sharing similarities in their extracellular and intracellular domains, in particular, the conserved intracellular Toll/IL-1 receptor (TIR) domain (Brikos & O'Neill, 2008; Watters *et al.*, 2007). Dimerization of TLRs induced by interaction with the corresponding ligands initiates the intracellular signalling cascade, followed by recruitment of intracellular adaptor proteins to their dimerized intracellular domains (Jin & Lee,

2008). Among the adaptor proteins, myeloid differentiation primary response gene (MyD88) and TIR-domain-containing adaptor- inducing interferon-β (TRIF) are critical for signalling, and hence, TLR signalling falls into the MyD88-dependent and TRIF-dependent pathways (O'Neill & Bowie, 2007; Yamamoto *et al.*, 2002), both terminating in activation of the transcription factors nuclear factor kB (NFkB) and activator protein-1 (AP-1). Binding to each TLR induces common effects, such as induction of inflammatory cytokines and chemokines and more specific and restricted activities, such as the induction of interferon-β (IFN-β). In turn, IFN-β amplifies the IFN response and the development of antiviral activity, thus contributing to an important defense against viral infections (Kaisho & Akira, 2006). The transcription factors interferon regulatory factor-3 (IRF-3) and interferon regulatory factor-7 (IRF-7) are master regulators of type I IFN activation and participate in both the TLR-dependent and independent pathways of innate immune responses to viral pathogens (Hiscott, 2007). Type I IFNs not only induce an antiviral state in most cells but also have diverse functions in the development of adaptive immunity.

The existence of several mechanisms in the immunocompetent host to avoid infections by pathogens has provided the selective pressure for successful viral pathogens to acquire strategies during evolution to evade the host immune responses (Alcami & Koszinowski, 2000), in particular for inhibiting and regulating TLR function, with several targeting the MyD88-dependent pathway (Bowie & Unterholzner, 2008). Viral infections are frequently sensed by TLR3, which detects dsRNA produced during replication of both DNA and RNA viruses. MyD88-independent signalling through the TLR3 pathway is initiated by the recruitment of the adaptor TRIF, leading to the subsequent production of inflammatory cytokines and type I IFNs and the up-regulation of co-stimulatory molecules. The importance of TLR3 signalling is emphasized by the fact that it is a target for immune evasion by viruses (Vercammen *et al.*, 2008), for example vaccinia virus A52R Malone (Bowie *et al.*, 2000; Harte *et al.*, 2003; Maloney *et al.*, 2005) and A46R (Bowie *et al.*, 2000; Stack *et al.*, 2005) proteins or hepatitis C virus (HCV) NS3/4A protease (Li *et al.*, 2005a, b). Downstream of TRIF, Sterile-alpha and Armadillo motif containing protein (SARM), another cellular molecule with a TIR domain, provides an important regulatory point in the MyD88-independent pathways (Carty *et al.*, 2006). Viruses may benefit from disrupting TLR signalling, and indeed it has been suggested that vaccinia virus directly engages TLRs (Yang *et al.*, 2004; Zhu *et al.*, 2007). Taking into account the complexity of pathogen recognition receptor (PRR) signalling in the innate immune response, it is

likely that viruses have evolved a whole range of mechanisms targeting different PRRs and at different levels of their signalling pathways. Thus, the study of virus evasion strategies is not only directly relevant to understanding virus pathogenesis but can also define novel aspects of our own immune system. For example, in HCV infection, the viral NS3/4, a protease complex targets and cleaves the interferon promoter stimulator-1 (IPS-1) adaptor protein to ablate signalling of immune defenses controlled by IFN a/b. Similarly, the finding that cleavage of IPS-1 by NS3/4 releases it from the mitochondrial membrane revealed that the mitochondrial attachment of IPS-1 is functionally important (Li *et al.*, 2005b). Here, we report a successful search for an ASFV host evasion gene that inhibits an important component of the innate immune response controlled by a TLR. A combination of bioinformatics, biochemical and reporter gene assays identified the ORF I329L, a late-expressed protein (Rodriguez *et al.*, 1992), which was characterized as a type I transmembrane protein with homology to TRIF in the C-terminal region and shown to be capable of inhibiting the TLR3 signalling pathway through an as yet undefined effect on the critical intracellular signalling adaptor molecule TRIF.

3 Materials and methods

3.1 Bioinformatic analysis

Full-length sequences of non-assigned ASFV ORFs were screened for homologies using protein-protein BLAST from the National Center for Biotechnology Information (NCBI). Screening for patterns or domains was performed using InterProScan 17.0 from the European Molecular Biology Laboratory (EMBL) and Prosite 20.33 databases. The transmembrane region prediction was performed by TransMembrane Helix prediction using the Hidden Markov Models 2.0 (TMHMM) program (Sonnhammer *et al.*, 1998), and 3D-structure similarities were analyzed with Protein Homology/analogy Recognition Engine (Phyre) (Bennett-Lovsey *et al.*, 2008). Multiple sequence alignments were made with ClustalW2 from the European Bioinformatics Institute (EBI) website.

3.2 Plasmids

Plasmids pcDNA$_3$-HA and pHR-CMV-eGFP were gifts from Dr. L. Dixon (Institute for Animal Health, Pirbright, UK) and Dr. Y. Ikeda (University College London, London, UK), respectively. The pFlag-TRIF- and pCCL5-promoter-containing plasmids were gifts from Dr. Andrew G. Bowie (Trinity College, Ireland), pDsRed-Monomer-Golgi and pDsRed2-ER vectors were purchased from ClonTech (catalogue number 632480 and 632409, respectively). The β-gal- CMV- (β-gal), the NFkB- (pPRD2) and IRF3- and IFN-β- promoter-containing plasmids were gifts from Dr. S. Goodbourn (St George's University, London).

The plasmids expressing TLR3 and CD4-TLRs chimaeras were provided by Dr. R. Medhzitov (Yale University, USA). Cloning of I329L, the ASFV ORF I329L (accession number NP_042833) was obtained by PCR amplification from DNA of the tissue-culture-adapted non-pathogenic ASFV isolate Ba71V (Uniprot database, code I329_ASFB7) with primers incorporating restriction sites for BamHI upstream and EcoRV downstream of the ORF and using the high-fidelity enzyme Pfu DNA polymerase. The primers used were 5'caggatccatgc- taagggttttcatatttttg3', 5'ctgatatcctttcttcttgaacatgaaacc3'. The amplified product was identified with ethidium bromide visualization on an agarose gel based on the expected size of the amplicon, and the DNA was purified from the excised band. The fragment was then cloned into plasmid pcDNA$_3$-HA, fused in frame with an N-terminal influenza

haemagglutinin (HA) peptide tag. The fidelity of the sequence of the cloned fragment was confirmed by automated sequencing.
For construction of a recombinant Lentivirus vector (pHR-CMV-HAI329L-eGFP), the I329L gene was excised from $pcDNA_3$, together with the HA tag, and cloned into the vector pHR-CMV-eGFP upstream of an internal ribosome entry site (IRES)-driven enhanced green fluorescent protein gene (eGFP).

3.3 Cells

Human HEK-293T and murine NIH-3T3 cell lines were cultured in 5% CO_2 at 37°C in Dulbecco's modified Eagle medium (DMEM) with GlutaMAXTM-I and 1000mg/l glucose, supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) and 100U/ml and 100µg/ml penicillin and streptomycin, respectively (all products from Invitrogen). The stable cell line 293-hTLR3-HA (HEK- TLR3), purchased from InvivoGen®, was maintained under the same conditions and cultured in regular DMEM supplemented with 10lg/ml blasticidin (Marshall-Clarke *et al.*, 2007).

3.4 Lentivirus production

Lentivirus was produced by transient transfection of HEK- 293T cells with a weight ratio of 3:1:1 of vector to packaging to envelope plasmids, respectively, using FuGENE 6 (Roche) according to the manufacturer's instructions. Control Lentivirus was produced by cotransfection of the packaging and envelope plasmid together with the empty pHR-CMV-eGFP plasmid. For production of recombinant Lentivirus-expressing I329L, the plasmid pHR-CMV-HA-I329L-eGFP was used.
Supernatants containing the Lentivirus were collected at 48h and 72h post-transfection and clarified by centrifugation, and the Lentivirus was collected by ultracentrifugation (125,000 x *g*, 3h, 4°C). Virus pellets were ressuspended in fresh culture medium, frozen at -80°C and titrated by infection of HEK-293T cells with a dilution factor of 4. Analysis of lentivirus-infected cells was done by detecting eGFP-positive cells by flow cytometry at 48h post infection (p.i.). Lentivirus transduction of NIH-3T3 cells with I329L. The NIH-I329L and NIH-eGFP stable cell lines were produced by lentivirus infection of NIH-3T3 cells with the recombinant pHR-CMV-HA-I329L-eGFP and empty pHR-CMV-eGFP (prepared as described in the section ''Cloning of I329L''), respectively, using a multiplicity of infection (m.o.i.) of 10 in DMEM. Analysis of NIH-I329L to determine the percentage of transduced cells was done by detecting eGFP-positive cells by flow cytometry at 48 h post-infection (p.i.), and this was verified to be greater than 99%.

3.5 Antibodies

The antibodies used in this study were as follows: rat monoclonal high-affinity antibody (clone 3F10) against HA conjugated with horseradish peroxidase (HRP) (Roche, 12013819001), rabbit polyclonal affinity-purified antibody against HA for immunoprecipitation assays (Sigma H6908), rat monoclonal high-affinity antibody (clone 3F10) against HA for immunofluorescence assays (Roche, 11867423001), donkey affinity-purified anti-rat IgG antibody conjugated with AMCA (Jackson Immuno Research, 712-155-150), mouse monoclonal antibody (clone B-5-1-2) against α-tubulin (Sigma-Aldrich, T6074) and rabbit polyclonal anti-mouse Immunoglobulins conjugated with HRP (Dako, P0161).

3.6 Immunoblotting

NIH-I329L cells were cultured in 75-cm^2 flasks at 37^{o}C, under 5% CO_2, in DMEM with GlutaMAXTM-I and 1000mg/l glucose, supplemented with 10% (v/v) heat- inactivated FCS and 100U/ml and 100µg/ml penicillin and streptomycin, respectively (all products from Invitrogen). Cells were lysed in cold lysis buffer (6M urea, 75mM NaCl, 1mM EDTA, 1% (v/v) NP40, 2% (v/v) glycerol, 1mM PMSF, 1µM DTT, 25 mM HEPES, pH 7.4). After protein quantification by the Bradford method using the Bio-Rad Protein Assay reagent, extracts were boiled (5min, 100^{o}C) with sample buffer (1.7% [w/v] SDS, 5% [v/v] glycerol, 0.1M DTT, bromophenol blue [0.02 mg/ml], 58mM Tris-HCl, pH 6.8). Proteins were then separated by 10-12% SDS-PAGE and electroblotted onto polyvinylidene fluoride (PVDF, BioRad) membranes and blocked with PBS-5% (w/v) non-fat dried milk for 1h at room temperature (RT). The development of the blots to reveal recombinant viral proteins was performed with a rat monoclonal high affinity antibody against HA conjugated with HRP (Roche) by incubating for 1h at RT or overnight at 4^{o}C, and the identified molecules were revealed with ECL chemiluminescent reagents (PerBio Science) according to the manufacturer's instructions. Blots were also developed with a mouse monoclonal antibody against α-tubulin (Sigma-Aldrich), followed by rabbit polyclonal anti-mouse immunoglobulins-HRP (Dako) to provide an internal control for protein loading. Immunoprecipitation Mouse NIH-I329L and mouse NIH-eGFP cells were collected by scraping from 75-cm^2 flasks and lysed using cold RIPA buffer (6M Urea, 75mM NaCl, 1mM EDTA, 1% (v/v) NP40, 2% (v/v) glycerol, 1mM PMSF, 5.5µg/ml protease inhibitors (SIGMA) and 1µM DTT, 25mM HEPES, pH 7.4) (1ml per 7x10^6 cells). The resulting protein extract was centrifuged at 20,000 x *g* for 10min at 4^{o}C to remove

cellular debris. The supernatant of the protein extracts was collected and, after determining the concentration of protein, was pre-cleared by adding 100µl of protein G beads. After rotating for 1h at 4°C, the beads were removed by centrifugation, and the recombinant I329L was recovered from the supernatant by adding protein G beads (100µl) and 5µg of rabbit polyclonal affinity-purified anti-HA antibody (Sigma). Following rotation for 1.5h at 4°C, the beads were then washed twice with lysis buffer without NP-40 and urea, and finally heated at 100°C for 10min with loading buffer (2% [w/v] SDS, 2% [v/v] β-mercaptoethanol, 0.1% [w/v] bromophenol blue, 10% [v/v] glycerol, 50mM Tris-HCl, pH 6.8) for 10min, and centrifuged for 30sec. The supernatants were then loaded onto an SDS-PAGE gel (10%).

3.7 Cell-surface biotinylation

Cell-surface biotinylation was done using a kit purchased from Pierce (#21335). One 75-cm^2 flask with transduced NIH-I329L cells was cultured until the cells reached 95% confluence. Then, the cells were washed twice with cold PBS and EZ-Link Sulfo-NHS-LC-Biotin dissolved in cold PBS (0.5mg/ml) was added to the cells, followed by incubation on a rocking platform with gentle agitation (30min, 4°C). To stop the reaction, 500µl of a ‘‘quenching’’ solution (supplied by the manufacturer without information) was added to each flask. Cells were then scraped off, centrifuged, washed with TBS and lysed in modified RIPA buffer (1% [v/v] Triton X-100, 0.2% [w/v] Na-deoxycholate, 150mM NaCl, 1 mM EDTA, 0.2% (w/v) SDS, 1mM PMSF, protease inhibitors, 50mM Tris-HCl, pH 7.4). The biotinylated surface proteins were recovered from the detergent lysate with streptavidin beads (Pierce Kit, #89881) (overnight, 4°C, in an orbital shaker). The streptavidin beads were centrifuged, washed 3 times with lysis buffer without detergent, and finally suspended in 100µl 1x loading buffer (2% [w/v] SDS, 2% [v/v] β-mercaptoethanol, 0.1%[w/v] bromophenol blue, 10%[v/v] glycerol, 50 mM Tris-HCl, pH 6.8). The samples were heated (10min, 100°C) and centrifuged, and the supernatants were loaded onto 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane (Bio-Rad) and blocked with 5% non-fat dried milk (1h, RT). The biotinylated HAI329L protein was revealed with a rat monoclonal high-affinity antibody against HA conjugated with HRP (Roche), followed by ECL chemiluminescent reagents (PerBio Science) according to the manufacturer’s instructions.

3.8 Deglycosylation assay

Lentivirus-transduced NIH-I329L cells were collected, and the recombinant I329L was immunoprecipitated as described above. Bound I329L was eluted from the anti- HA protein G beads by heating (10min, 100°C) in 0.5% (w/v) SDS with 10% (v/v) β-mercaptoethanol. For deglycosylation, the eluted sample was treated with peptide-N glycosidase F (PNGaseF) (Roche) or endoglycosidase H (Endo H) (New England Biolabs) according to the manufacturer's instructions. As controls, the enzymes were omitted. Proteins were then precipitated with ethanol at 80% (v/v) (overnight, -20°C). The pellet was ressuspended in 20µl of SDS-PAGE loading buffer, heated (10min, 100°C), and loaded onto an SDS-PAGE gel.

3.9 Immunofluorescence to demonstrate co-localization of I329L with ER and Golgi

NIH-I329L cells were seeded onto coverslips (pre-treated with 1% [v/v] poly-L-lysine) and incubated overnight at 37°C and 5% CO_2. For double staining to reveal the endoplasmatic reticulum and the transmedial region of the Golgi apparatus, the cells were transfected using Lipofectamine 2000 (Invitrogen) with 2µg of a plasmid coding for the ER retention sequence KDEL (plasmid pDsRed2-ER, Clontech) or coding for a sequence corresponding to the N-terminal 81 amino acids of human beta-1, 4-galactosyltransferase (GT) (plasmid pDsRed-Monomer-Golgi, Clontech) fused with the fluorescent red protein. After transfection for 24h, the cells were fixed with 4% (w/v) paraformaldehyde in PBS for 20min at RT and then incubated in a blocking solution (PBS containing 0.05% Tween-20 and 5% normal goat serum) (30min, RT). The cells were permeabilized using PBS containing 0.1% (v/v) Triton X-100. To detect recombinant I329L, a primary rat monoclonal high-affinity antibody against HA diluted in blocking solution was used (1h, RT), followed by a donkey affinity-purified anti-rat IgG antibody conjugated with AMCA (1h, RT). All washes after incubation with antibody were performed at RT with PBS containing 0.05% (v/v) Tween-20. Cells were mounted in Vectashield (Vector Laboratories) and observed under a fluorescence microscope. HA-I329L is shown with a green colour for better visual perception. Fluorescent images were taken with a Leica DMRA2 microscope equipped with a cooled CCD camera (Leica. Corp., Wetzlar, Germany).

3.1.0 Luciferase reporter assays

For the CD4-TLR reporter gene assay, HEK-293T cells were cultured in 24-well plates at $6x10^4$/well, and 24h later, they were transfected using Lipofectamine 2000 (Invitrogen) with three plasmids. One, the NFkB-driven luciferase reporter contained only the positive regulatory domain, PRD2, of the NFkB binding site of the IFN-β promoter (NFkB) (100ng), the second contained β-gal-CMV (β-gal) (25ng), and the third chimeric plasmid contained a CD4 extracellular domain co-ligated upstream of a TLR1 (CD4-TLR1) or TLR3 (CD4-TLR3) intracellular domain (50ng). The effect of the co-transfection of the I329L gene cloned into the $pcDNA_3$ plasmid ($pcDNA_3$-I329L) (300ng) provides an assay for interference of the viral protein with the TLR signalling pathway (Medzhitov *et al.*, 1997). Empty $pcDNA_3$ served as the negative control ($pcDNA_3$). After incubating for 48h at 37°C, the cells were lysed in 100µl lysis solution (ABX210LM, Promega Systems) according to the manufacturer's instructions, and samples were assayed for both luciferase and β-galactosidase activities. The luciferase activity was normalized to the β-galactosidase activity from the co-transfected plasmid internal control and expressed as luciferase relative to galactosidase activity. Values are expressed as mean relative stimulation ± SD (calculated from triplicate determinations). A minimum of three independent assays was done for each experiment reported. In similar reporter assays, HEK-TLR3 cells were transfected with reporter plasmids for IFN-β, NFkB, CCL5, and the internal control β-gal, and expression plasmids $pcDNA_3$-I329L or $pcDNA_3$ (negative control), keeping the total amounts of DNA and equimolar ratios constant in all assays by adding the appropriate amount of empty vector. After 48h, cells were stimulated with polyinosinic: polycytidylic acid (poly (I:C)) or IL1-β (PeproTech) or TNF-α (PeproTech) as indicated in the figures for 5 hours. The cells were lysed and assayed for luciferase as described above. IFN-β reporter assays were also done using HEK 293T cells ectopically expressing TRIF. Transfections were performed with equal amounts of DNA mixtures comprising 25ng of TRIF plasmid vector (TRIF), the I329L plasmid vector ($pcDNA_3$-I329L) (0, 100, 200 and 300ng), the IFN-β-reporter plasmids (IFN-β) (100ng) and the β-gal internal control plasmid (β-gal) (25ng). The $pcDNA_3$ negative control was included in the transfection whenever necessary in order to keep the total amounts of DNA and equimolar ratios constant in all assays. After 48h, the cells were stimulated for 6 hours with poly (I:C) (25µg/ml) where indicated, before harvesting the cells. Lysis and luciferase activity were measured and normalized to the β-galactosidase activity as described above. Finally, to determine the impact of intracellular stimulation with poly (I:C), HEK-293T cells

were transfected with 300ng empty vector ($pcDNA_3$) or 300ng I329L plasmid vector ($pcDNA_3$-I329L) together with 100ng of IFN-β plasmid (IFN-β). After 48h, the cells were stimulated for 6h with intracellularly delivered poly (I:C) (0.5µg/ml) using Lipofectamine 2000 (Invitrogen). Lysis and luciferase activity were measured and normalized to the β-galactosidase activity as described above.

3.1.1 Statistical analysis

Data are expressed as mean ± SD and were analyzed for significance using Student's T test. P-values are as follows: *** $P<0.005$, ** $P<0.01$ and * $P<0.05$.

4.0 Results

4.1 Identification of ASFV ORF I329L as a potential homologue of the TLR family

Bioinformatic analysis of the sequence of pI329L from the tissue-culture-adapted non-pathogenic ASFV isolate Ba71V (Uniprot database code I329_ASFB7), which grows in a variety of commonly used fibroblast cell lines, reveals a type I membrane structure with a 17-amino-acid signal peptide (amino acids 1-17), followed by an N-terminal extracellular domain (amino acids 18-239), a transmembrane domain (amino acids 240-260), and a 69-amino-acid C-terminal intracellular domain (amino acids 261-329).

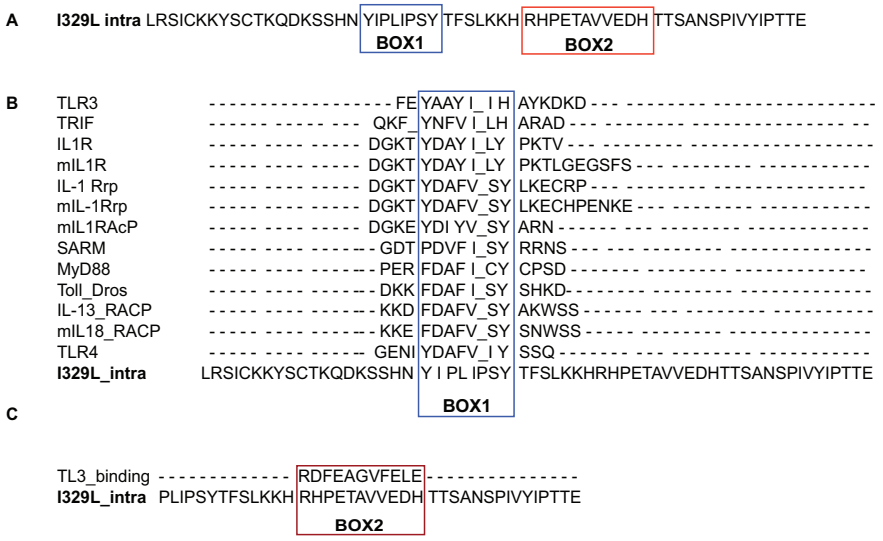

Figure 1. Homology with TLR3 in the intercellular domain of ASFV protein I329L. The analysis presented here was performed with ClustalW2. **1A.** Sequence of the intracellular domain of I329L (amino acid residues 260 to 329) indicating the predicted BOX1 and BOX2 regions. **1B.** Predicted TIR-like domain in the BOX1 domain of I329L has sequence homology with known proteins containing the BOX1 TIR domain. **1C.** Predicted TIR-like domain in the BOX2 domain of I329L.

A number of potential glycosylation sites were indicated, at amino acids 32, 39, 44, 76, 82, 101, 185, and 219. Simple sequence examination of the sequence database did not show any gene similar to I329L; however, after multiple alignments with several TLR proteins, a weak but interesting alignment between I329L and TLR from Drosophila melanogaster was revealed. Importantly, the intracellular domain of I329L aligned with BOX1 and BOX2 regions of the human TIR-like domain of TLR3 **(Figure 1A)**, with a sequence similarity of 35% to TLR3

in BOX1 **(Figure 1B)**. Furthermore, the BOX2 of TLR3 also showed similarities with the I329L intracellular domain **(Figure 1C)**. Finally, the conserved extracellular region with leucine-rich repeats (LRRs) is an important motif for interaction between proteins and is common to several TLRs. Although the observed similarity was admittedly weak, we nonetheless decided to clone and test I329L for its impact on poly (I:C)-stimulated innate immunity in reporter assays (see below).The cellular distribution of ASFV ORF I329L is consistent with ER, Golgi and plasma membrane localization Bioinformatic analysis and demonstration of extensive glycosylation suggested that the I329L protein was a typical transmembrane protein. This possibility was confirmed in I329L-lentivirus-transduced NIH-3T3 cells (NIH-I329L) by immunofluorescence and cell-surface labelling. For immunofluorescence of I329L, the I329L protein was detected with a rat monoclonal high-affinity antibody (clone 3F10) against HA, followed by a donkey affinity- purified anti-rat IgG antibody conjugated with AMCA. Co-detection of the endoplasmic reticulum (ER) and Golgi apparatus was achieved by simultaneously transfecting the cells with two marker plasmids: one coding for red-fluorescent protein (dsRed) fused with the KDEL sequence in order to target ER (plasmid pDsRed2-ER) and the other coding for dsRed fused with an integral protein of the Golgi apparatus (plasmid pDsRed-Monomer-Golgi). As can be seen, the cellular distribution of I329L corresponds to that of the markers for ER and Golgi **(Figure 2A and 2B)** , indicating that I329L passes through the secretory pathway. Finally, since the intracellular secretory pathway frequently culminates the incorporation of glycoproteins into the cell membrane, viable NIH-I329L-transduced cells were surface-biotinylated. Subsequent immunoprecipitation with streptavidin and western blotting with anti-HA to detect I329L confirmed the surface expression of I329L in the transduced cells **(Figure 2D)**.

4.2 ASFV ORF I329L codes for a highly glycosylated protein

Expression of HA-I329L in NIH-3T3 cells was accomplished by transduction with HA-I329L recombinant Lentivirus (NIH-I329L). The protein immunoprecipitated with anti-HA antibody migrated in SDS-PAGE with an apparent molecular weight of *50 kDa, significantly larger than the molecular weight predicted from the amino acid sequence alone **(Figure 2C**, **lanes b and d)**, thus raising the possibility of extensive glycosylation, as indeed is suggested by bioinformatic predictions.
Therefore, the recombinant I329L protein was immunoprecipitated from NIH-I329L cells and digested with two endoglycosidases, PNGase F, which digests high-mannose, hybrid and complex types of oligosaccharides, and endoglycosidase H (EndoH), which digests

only high-mannose and hybrid types of oligosaccharides. Digestion with EndoH and PNGase F yielded recombinant proteins that migrated with a molecular mass of *37 kDa **(Figure 2C, lande a)**, and *36 kDa **(Figure 2C, lane c)** , respectively, thus confirming that the I329L protein is extensively glycosylated. Furthermore, the molecular weight observed for the sample after digestion with PNGase F was lower than after digestion with Endo H, suggesting that I329L has a small number of glycans of the complex type.

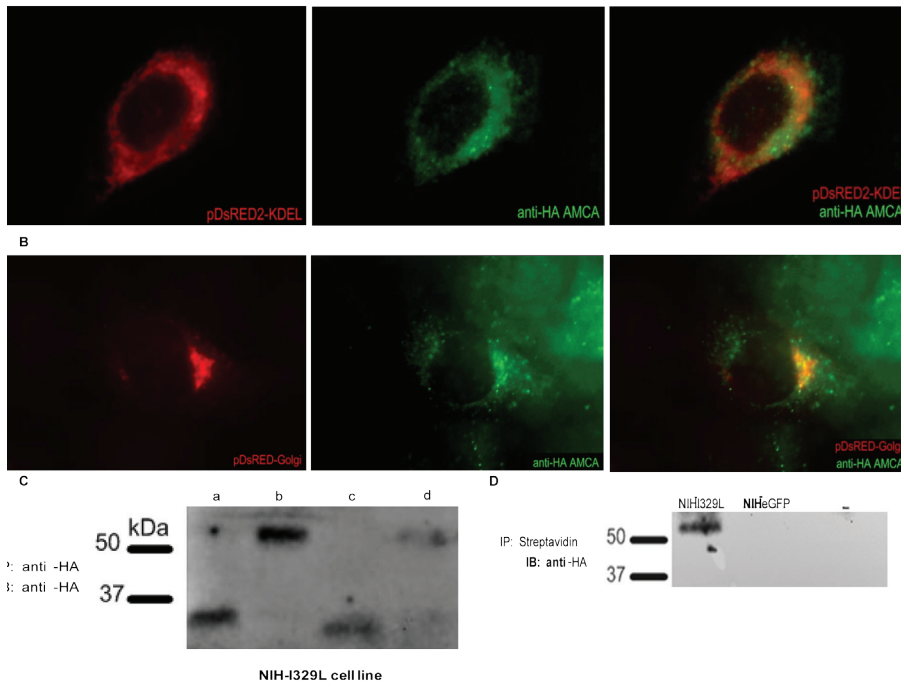

Figure 2. ASFV protein I329L is higly glycosylated and localizes to cell membranes.

2A. I329L protein distribution is consistent with endoplasmic reticulum localization. Lentivirus-transduced NIH-I329L cells were transfected with plasmid pDsRed2-ER to localize the protein to the ER (red, left) and then co-stained with a rat monoclonal high-affinity antibody against the HA, followed by a donkey affinity-purified anti-rat IgG antibody conjugated with AMCA (left, middle). The colocalization is evident in the merged image (right panel). **2B.** I329L protein distribution is consistent with Golgi localization. The NIH-I329L cells were transfected with plasmid pDsRed-Monomer-Golgi to localize the protein to the Golgi (red, left) and then co-stained with a rat monoclonal high-affinity antibody against the HA, followed by a donkey affinity purified anti-rat IgG antibody conjugated with AMCA (left, middle). The colocalization is evident in the merged image (right panel). **2C.** The I329L protein was immunoprecipitated from lysates from the lentivirus-transduced NIH-I329L cell line with rabbit anti-HA antibodies bound to protein G beads. The bound protein was eluted and digested with EndoH (a) or PNGase (c) and examined by SDS- PAGE. Controls samples were treated with only EndoH buffer (b) or only PNGase buffer (d). **2D)** I329L protein is expressed at the cell surface. Biotinylated surface proteins from biotynylated NIH-I329L cells were collected with streptavidin beads, loaded onto a 10% SDS-PAGE gel and transferred to PVDF membranes, and I329L was revealed with a rat monoclonal antibody against HA conjugated with HRP (band in lane NIHI329L on the left). Lane NIHeGFP represents NIH-3T3 cells transduced with the empty lentivirus.

4.3 ASFV ORF I329L inhibits TLR3 signalling

An important sensor of viral infections is TLR3, which detects dsRNA and signals through a MyD88-indepen- dent pathway. In order to demonstrate that I329L was interfering with TLR3 signalling, we used two entirely different experiments. In the first approach, we used a CD4-TLR assay focusing on an NFkB-driven luciferase reporter. These CD4-TLR constructs comprise the murine CD4 extracellular domain co-ligated to the cytoplasmatic domain of a human TLR. The extracellular CD4 domain dimerizes spontaneously, resulting in dimerization of the intracellular TLR domain and thus constitutive activation of NFkB, which is detected using the simultaneously transfected luciferase reporter plasmid. Using this system, expression of I329L was found to inhibit the induction of the NFkB-dependent reporter gene signal **(Figure 3A)**. Negative controls consisted of plasmid $pcDNA_3$ coding for H171R, a known structural viral protein of ASFV, instead of the I329L gene, and a CD4-TLR1 construct instead of CD4-TLR3 **(Figure 3A)**. As the synthetic dsRNA analogue polyinosine-poly-cytidylic acid (poly (I:C)) activates cells via TLR3, in the second system, we also tested the effect of I329L on TLR3-dependent NFkB activation induced by poly (I:C). Cells (HEK-293T) were co-transfected with a construct encoding TLR3, the luciferase reporter containing the NFkB (PRD2) binding sequence, and the plasmid containing the cloned ASFV I329L gene. The cells were stimulated with increasing concentrations of poly (I:C) (0, 5, 25, and 50ug/ml). The vector of the I329L gene without an insert ($pcDNA_3$) was used as negative control.

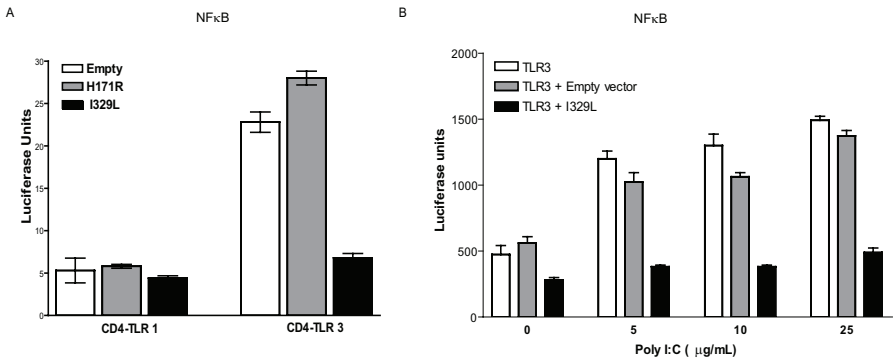

Figure3. I329L inhibits NFkB activation by TLR3.
3A. I329L inhibits activation of NFjB. HEK-293T cells were transfected with constitutively active CD4-TLR plasmids (50ng CD4-TLR1 or CD4-TLR3) in the presence of 300ng of I329L plasmid (pcDNA$_3$-I329L), 100 ng of NFkB reporter plasmid (NFkB) and 25ng of β-gal reporter plasmid (β-gal) (black bars) and incubated for 48 h prior to reporter assays. The negative controls were the plasmid vector without insert (pcDNA$_3$) (white bars) or with the control H171R insert (pcDNA$_3$- H171R) (grey bars). **3B.** I329L inhibits poly (I:C)-dependent activation of NFkB. HEK-293T cells were co-transfected with plasmid vectors encoding 50 ng TLR3 (TLR3), 100ng NFkB reporter plasmid (NFkB), 300 ng ASFV I329L gene (pcDNA$_3$-I329L) and 25ng of β-gal reporter plasmid (β-gal) (black bars). Negative and positive controls were the plasmid vector of I329L gene without an insert (pcDNA$_3$) and TLR3 (grey bars), and the TLR3 plasmid alone (white bars). The cells were stimulated with poly (I:C) at the concentration indicated. Luciferase activity was normalized to the β-galactosidase activity obtained with the co-transfected β-gal plasmid internal control. Standard deviations are shown by error bars.

This TLR3-dependent activation of NFkB in HEK-293T cells expressing TLR3 was inhibited by the ASFV gene I329L at all concentrations of the ligand, poly (I:C), that were tested **(Figure 3B)**. Thus, I329L was shown by two independent approaches to inhibit the TLR3-mediated activation of NFkB.

4.4 The I329L gene mediates MyD88-independent induction of poly (I:C)-stimulated activation of NFkB and IRF3

To confirm and further explore the mechanisms of I329L-mediated inhibition of TLR3 activation, human embryonic kidney fibroblasts (HEK-293T) stably expressing TLR3 (HEK-TLR3) were transfected together with an NFkB luciferase reporter plasmid (NFkB) and stimulated with poly (I:C) or IL-1β. As can be seen, the poly (I:C)-stimulated activation of NFkB via the established TLR3 pathway was inhibited by expression of I329L **(Figure 4A)**. Activation of NFkB can occur either dependently or independently of the MyD88 adaptor molecule.

However, the absence of an effect on the IL-1 receptor pathway excludes the possibility that I329L modulates TLR signalling by interacting with the adaptor molecule MyD88. Finally, we failed to find any inhibitory effect of I329L on NFkB activation stimulated by TNF-α, indicating that I329L does not have impact on the NFkB activation pathway via molecules other than those downstream of TLR ligation. Taking these results together suggests that I329L acts upstream of the IkB kinase complex, which in the case of TLR3 signalling could be TRIF, a possibility explored below.

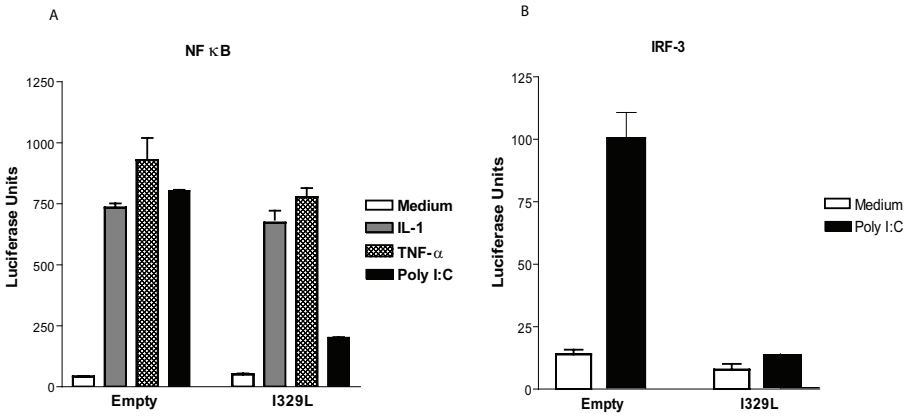

Figure 4. I329L inhibits NFkB and IRF3 activation through a MyD88-independent pathway.

4A. I329L inhibits activation of NFkB via Poly I:C but not TNF-α or IL-1. HEK-TLR3 cells were co-transfected with 300ng of empty plasmid vector ($pcDNA_3$) or 300ng of the same plasmid vector encoding I329L ($pcDNA_3$-I329L), together with 100ng of the NFkB luciferase reporter plasmid (NFkB) and 25ng of β-galactosidase reporter plasmid (β-gal), and cultured for 48h. At 6h before harvesting, cells were not stimulated (white bars), or stimulated with IL-1β (100ng/ml) (grey bars), TNF-α (100ng/ml) (hatched bars) or poly (I:C) (25μg/ml) (black bars). The NFkB reporter gene activity was then measured. **4B.** I329L inhibits activation of IRF3. HEK-TLR3 cells were transfected with 300ng empty vector ($pcDNA_3$) or 300ng of the same plasmid encoding I329L ($pcDNA_3$-I329L) together with 100ng of IRF3 (IRF3) and 25ng of β-galactosidase reporter plasmids (β-gal), and 6h before harvesting, cells were stimulated extracellularly with 25μg/ml poly (I:C). Luciferase activity was normalized to the β-galactosidase activity given by the co-transfected β-gal plasmid internal control. Standard deviations are shown by error bars.

4.5 Signal transduction initiated through TLR3 ultimately diverges into two signalling branches, leading to activation of NFkB and IRF3.

In order to determine whether I329L also inhibits activation of IRF3, HEK-TLR3 cells were co-transfected with I329L ($pcDNA_3$) and a reporter plasmid containing the promoter of IRF3 (IRF3) and then stimulated with poly (I:C). As can be seen **(Figure 4B)**, in the presence of I329L, activation of the IRF3 promoter was significantly reduced. I329L ASFV ORF inhibition of activation of IRF3 and subsequent expression of IFN-β and CCL5 responses. As TLR3 activates and programs interferon and chemokine expression through the coordinated activation of IRF3 and NFkB (Melchjorsen *et al.*, 2003; Takeuchi *et al.*, 2004), we next examined the effect of I329L on the activation of CCL5 expression using an appropriate reporter plasmid. We also extended the previously presented I329L-mediated inhibition of the NFkB-restricted PRD2 by using a reporter plasmid containing the entire IFN-β sequence. The rationale for this was that inhibition of activation of NFkB and IRF3 is predicted to inhibit induction of IFN-β and chemokine responses, two major effectors of the antiviral response induced by TLR3. As can be seen, poly (I:C)-induced CCL5 activation from HEK-293T cells stably expressing TLR3 (HEK- TLR3) was inhibited by I329L **(Figure 5A)**. Moreover, poly (I:C)-induced IFN-β activation was also inhibited by I329L in HEK-TLR3 cells **(Figure 5B)**. Both results are consistent with an I329L-mediated inhibition of a MyD88-independent activation pathway (Fitzgerald *et al.*, 2003) and raise the possibility that I329L targets TRIF, as TRIF is the only known adaptor protein that interacts with TLR3 and mediates induction of IFN-β through activation of NFkB and IRF3. The CCL5 promoter is under the control of the PRD2 and ISRE domains (binding NFkB and IRF3, respectively), the cAMP responsive element (CRE), AP-1, the gamma interferon activation site (GAS), the nuclear factor of activated T-cells (NFAT) and CCAAT/enhancer-binding protein (C/EBP) domains (binding proteins c-Jun/activating transcription factor 2 (ATF2), Fos/c-Jun, signal transducer and activator of transcription (STAT), NFAT and C/EBPb. In our experimental system, the induction of CCL5 and IFN-β is the consequence of a poly (I:C)-stimulated MyD88-independent activation of TLR3. Thus, the possibility of a MyD88-dependent, IRF5-mediated activation of CCL5 transcription can be excluded.

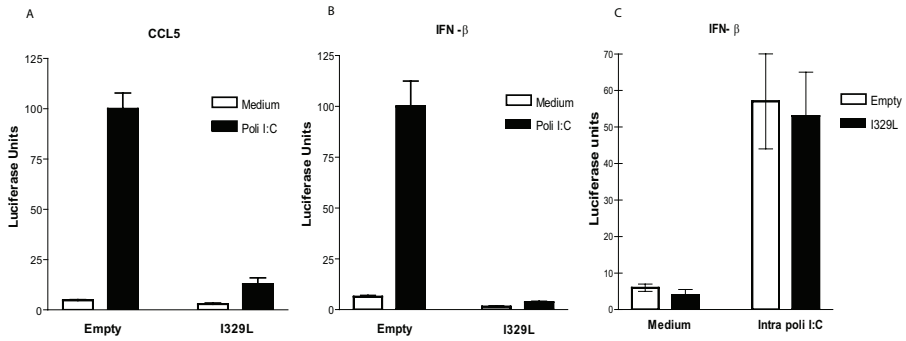

Figure 5. I329L inhibits activation of CCL5 and IFN-β promoter.
5A. I329L inhibits activation of CCL5. HEK-TLR3 cells were transfected with 300ng empty plasmid vector ($pcDNA_3$) or 300ng I329L plasmid vector ($pcDNA_3$-I329L) together with 100ng of CCL5 reporter plasmid vector (CCL5). 6h before harvesting, cells were stimulated extracellularly with 25µg/ml poly (I:C). **5B.** I329L inhibits activation of IFN-β. HEK-TLR3 cells were transfected with 300ng empty vector ($pcDNA_3$) or 300ng of I329L plasmid vector ($pcDNA_3$-I329L) together with 100ng of IFN-β plasmid vector (IFN-β). 6h before harvesting, cells were stimulated extracellularly with 25µg/ml poly (I:C). **5C.** I329L does not inhibit intracellular IFN-β activation independently of TLR3. HEK-293T cells were transfected with 300ng empty vector ($pcDNA_3$) or 300ng I329L plasmid vector ($pcDNA_3$-I329L) together with 100ng of IFN-β plasmid reporter (IFN-β). Six hours before harvesting, cells were stimulated intracellularly using Lipofectamine 2000 with 0.5µg/ml poly (I:C). Luciferase activity was normalized to the β-galactosidase activity obtained with the cotransfected β-gal plasmid internal control. Standard deviations are shown by error bars.

The possibility that I329L inhibits IFN-β activation induced intracellularly via a receptor other than TLR3 was investigated by introduction of poly (I:C) into HEK-TLR3 cells. Interestingly, there was no effect of I329L on IFN-β activation via an intracellular poly (I:C) stimulus (Lipofectamine-mediated poly (I:C) transfection) **(Figure 5C)**, and we therefore exclude the hypothesis that the effect of I329L is mediated via intracellular receptors such as melanoma differentiation associated gene 5 (MDA-5) or retinoic-acid- inducible protein I (RIG-1). Taken together, these results suggest that I329L inhibits induction of promoters that are known to contain NFkB or IRF3 binding sites via the TLR3 pathway and not via intracellular receptors like MDA-5 and RIG-I. Reversal of ASFV-ORF-I329L-mediated inhibition of the activation of NFkB and IFN-β promoters by overexpression of TRIF. Activation of the TLR3-TRIF signalling pathway not only induces cytokines, co-stimulatory molecules and antimicrobial peptides that are induced by all TLRs but also antiviral type I interferon and specific chemokines including IP-10 and CCL5 (Melchjorsen *et al.*, 2003; Takeuchi *et al.*, 2004). A direct effect of I329L on TRIF signalling was demonstrated using HEK-TLR3 cells transfected with TRIF as, in the presence of

I329L, the IFN-β signalling pathway was inhibited **(Figure 6A)**. To further investigate the possible impact of I329L on TRIF signalling, HEK-TLR3 cells were simultaneously transfected with I329L (pcDNA$_3$-I329L), the IFN-β luciferase reporter plasmid (IFN-β) and increasing quantities of TRIF plasmid (TRIF) and then stimulated with poly (I:C). As can be seen, overexpression of TRIF reversed the inhibition of reporter activation induced by I329L in a dose-dependent manner, consistent with the hypothesis that the ASFV gene I329L targets TRIF signalling **(Figure 6B)**.

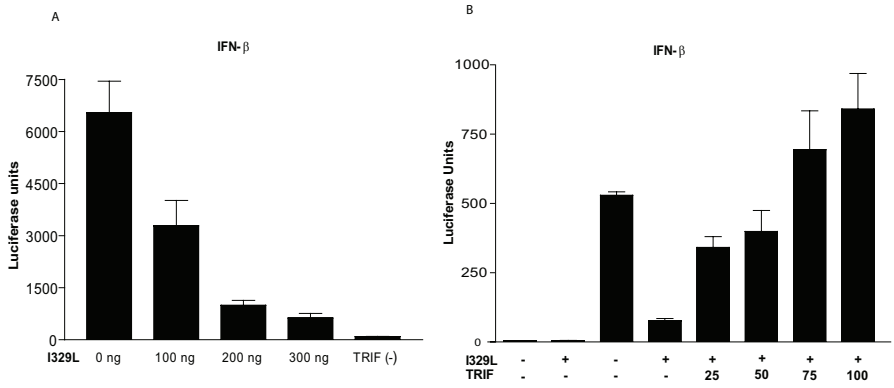

Figure 6. I329L inhibits activation of the IFN-β promoter at the level of TRIF.
6A. I329L inhibits TRIF-mediated activation of IFN-β. Activation of the IFN-β promoter reporter plasmid was induced via ectopic expression of TRIF in HEK-293T cells through transfection with the TRIF plasmid vector. Transfections were performed with equal amounts of DNA comprising 25ng of TRIF plasmid vector (TRIF) in the presence of increasing amounts of I329L plasmid vector (100–300ng) (pcDNA$_3$-I329L) jointly with IFN-β-reporter plasmids (IFN-β). **6B.** Overexpression of TRIF reverses I329L-mediated inhibition of IFN-β activation. TRIF was ectopically expressed in HEK-293T cells with increasing amounts of TRIF plasmid vector (25–100ng) (TRIF) in the presence of 200ng of the plasmid vector coding for I329L (pcDNA$_3$-I329L) simultaneously with 100ng of IFN-β-reporter plasmid vector (IFN-β) plus 25ng of β-gal plasmid vector (β-gal) and after stimulating for 6 hours with 25μg/ml of poly (I:C) where indicated, before harvesting the cells. In all cases, luciferase activity was measured after 48h. Luciferase activity was normalized to the β-galactosidase activity obtained with the cotransfected β-gal plasmid internal control. Standard deviations are shown by error bars.

5 Discussion

The necessity to recognize and destroy invading pathogens has played a crucial role in the evolution of the immunesystem of both vertebrates and invertebrates. At the same time, pathogens and, in particular, viruses have evolved reciprocal strategies to manipulate the immune system. Here, we describe a novel gene from African swinefever virus (ASFV), I329L, which is able to modulate one of the most critical steps of the innate immune response by interfering with TLR3-stimulated activation. This may constitute an advantage for the virus with an impact on both its vertebrate and invertebrate hosts, pigs and ticks, respectively. Conventional bioinformatic analysis of the sequence of I329L predicted a putative type 1 transmembrane protein with an intracellular domain followed by a transmembrane region and an extracellular domain with nine potential glycosylation sites and leucine-rich repeats (LRRs). Both TLR3 and I329L are type I transmembrane proteins with 20% of their sequence intracellular and more than 70% in a highly glycosylated extracellular domain with LRRs. Interestingly, more detailed analysis revealed that the intracellular domain of I329L contains a region of homology with BOX1 and BOX2 of TLR3-TIR domain **(Figure 1A-C)**. In addition, the putative intracellular domain contains a number of intracellular signalling sequences such as SH2 and SH3 domain binding motifs, a major TRAF2- binding consensus motif and a CK2 phosphorylation site (data not shown). In the work mentioned above, we con- firmed that ORF I329L codes for a highly glycosylated protein **(Figure 2C)**. Significantly, TLR3 is one of the most heavily glycosylated of the TLRs (Bell *et al.*, 2006), and ORF I329L was demonstrated to be expressed in the cell membranes **(Figure 2A, B)** and at the cell surface **(Figure 2D)**, locations where many receptor and adaptor molecules involved in innate immunity have been identified (Fan *et al.*, 2008). Consistent with this, we showed that I329L inhibited double-stranded- RNA-stimulated activation of NFkB **(Figure 4A)** and IRF3 **(Figure 4B)**, two key players in the innate antiviral response. As might be predicted, expression of I329L protein also inhibited the activation of CCL5 **(Figure 5A)** and IFN-β **(Figure 5B)**. Finally, IRF3 activation mediated by expression of TRIF was inhibited by I329L **(Figure 6A)**, whereas over- expression of TRIF reverted the inhibition of reporter activation induced by I329L in a dose-dependent manner **(Figure 6B)**. Thus, I329L may be targeting at the level of TRIF, a key adaptor molecule in the MyD88-independent pathway. Direct biochemical proof of this has not been possible, despite many attempts. Although this is the first ASFV gene manipulating TLRresponses to be described, vaccinia virus has evolved two proteins, A46R and A52R, that are inhibitors of the TIR- mediated

immune response (Bowie & Unterholzner, 2008).
Based on our experimental data and the bioinformatic data presented above, some hypothetical models for the ASFV TLR-βased strategies to evade the host defence by targeting TLR3 were considered. The most immediate explanation focuses on the intracellular domain of I329L, with its abundance of signalling motifs in particular, the putative TIR homologous region and the reversion of I329L-mediated inhibition by overexpression of TRIF. The later experiment suggested that the cytoplasmic tail of I329L might interfere with recruitment of TRIF, an important adaptor protein involved in the MyD88-independent pathway. Similarly, a direct interaction between the cytoplasmic tail of I329L and TLR3 was suggested by the observed sequence homology between BOX1 of the TIR domain and the intracellular region YIPLIPSY sequence of I329L **(Figure 1B)**. This conclusion is supported by the experimental data showing I329L-mediated inhibition of IRF3 and NFkB activation and the predicted inhibition of induction of expression of CCL5 **(Figure 5A)** and IFN-β **(Figure 5B)** induced by externally, but not by internally, delivered poly (I:C) **(Figure 5C)**. Finally, the observation that increasing amounts of I329L were able to inhibit TRIF-mediated NFkB **(Figure 4A)** and IRF3 **(Figure 4B)** activation is consistent with the hypothesis that I329L acts at the level of TRIF in the signal transduction pathway. Thus, the possibility that I329L protein interacts with the specific intracellular area responsible for TLR3 homodimerization cannot be excluded, although in most but not all cells, TLR3 is localized to the endosome and not on the cell surface. It is worth adding that when the intracellular domain of ORF I329L is aligned to several TIR domains, it shows a higher homology to the TLR3-TIR domain than to other TIR-domain-containing molecules. The absence of I329L RNA in ASFV-infected cells until20 hours post infection, as shown by northern blot analysis (Rodriguez *et al.*, 1992), is difficult to reconcile with our findings; that is, a virus mechanism such as I329L, evolved to inhibit IFN and chemokine induction, might be predicted to be expressed early in virus infection. Thus, it will be important to measure expression of I329L by a method that is more sensitive than northern blotting. In conclusion, the results presented show that ORFI329L is able to impair the cellular responses controlled by TLR3 that lead to both IFN-β production and NFkB activation. The precise mechanism for this inhibition remains to be elucidated, but our current working hypothesis based on our observations and the current literature is that the ORF-I329L-mediated inhibition is intracellular and might be related to an interaction with TRIF. In view of the major impact of I329L on innate immunity, its deletion may provide a rational strategy for construction of an attenuated deletion mutant vaccine.

6 Acknowledgments

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03 | The Impact of B cell Restricted Transgenic Expression of a Mouse Herpes Virus (MHV-68) Host Evasion Gene

1 Abstract

To elucidate the role of the M2 protein of the MHV-68 virus *in vivo*, B lymphocyte restricted M2-transgenic mice were constructed. M2 has been implicated in the establishment, maintenance and reactivation of latency in B cells. Interestingly, M2 also interacts with the VAV signalling system, suggesting that it may modulate B-cell receptor-mediated signalling events through Vav to influence B-cell activation, proliferation and/or survival. The analysis of surface markers expression of these transgenic mice demonstrated that M2 transgene made no impact on the development of B cells, as there was no difference in the development of B-cell subsets, either in the bone marrow or in the spleen. However, transgenic mice immunized with the T dependent antigen (DNP-KLH) produced significantly higher levels of both IgM and IgG2a antibodies. This work shows that virus host evasion genes can be used as valuable tools to manipulate the genetic program of mammalian cells *in vivo*. Thus B-cell restricted transgenic expression of a virus host modification gene has interfered with the production of antibodies and the numbers of germinal centre.

2 Introduction

The availability of genetically modified mice lacking components of the innate and adaptive immune system, together with genetically manipulated MHV-68 viruses, has provided useful tools to explore the pathogenesis and the role of specific viral genes in the virus-host interaction. The construction of transgenic mice is also a powerful strategy to study mechanisms of immunity *in vivo*. The use of specific promoters to restrict cellular transgene expression to a defined cell type is an additional strategy, which may overcome the undesirable effects of whole body transgenesis. Interestingly, cell specific transgenic expression of virus host modulatory genes is an alternative approach to study the role of a viral protein in the context of the homologous virus infection. This system allows an evaluation of the function of a given virus gene expressed in a specific cell type in physiological conditions and in context of infection during acute and latent phases.

This work focuses on the role of the latency-associated M2 gene. The M2 open reading frame is located at the left end of the MHV-68 genome and shares positional homology with latency-associated genes in other gammaherpesviruses. Functional studies performed *in vitro* indicated that M2 is a latency-associated protein that has an important role in B cell activation, proliferation and survival (Jacoby *et al.*, 2002). *In vivo* experiments using mutant M2 viruses have shown that M2 protein is critical for establishing latency, following intranasal inoculation, and for virus reactivation, following intraperitoneal inoculation (Herskowitz *et al.*, 2005; Jacoby *et al.*, 2002). In the absence of M2, infected B cells are unable to efficiently transition from the germinal centre to the follicles (Simas *et al.*, 2004). Early in latency, there is an accumulation of latently infected naïve B cells in the absence of the M2 protein, indicating a role for the M2 protein in affecting B cell development during infection (Herskowitz *et al.*, 2005). B cell proliferation is necessary for the establishment of MHV-68 latency, and, similar to Epstein-Barr virus (EBV), memory B cells are the primary latency reservoir (Moser *et al.*, 2005; Willer & Speck, 2003). In addition, M2 protein correlates with high serum IL-10 levels and an increased frequency of virus-specific CD8+ T cells during MHV-68 infection (Siegel *et al.*, 2008). This is consistent with previous data that indicated that M2 is a major target for the host cytotoxic T lymphocyte response (Husain *et al.*, 1999).

This part of our work reports the construction of a B-cell restricted M2-transgenic mouse and focuses on the impact of the M2 transgene on the B cell physiology *in vivo*, analyzing the B cell compartment and the effect of different challenges in an M2-transgenic mouse and respective controls.

3 Materials and methods

3.1 Mice

FVB/N mice were bred and kept at the IGC. The transgenic M2 founder mice were produced at the Transgenic Facility of IGC by Dr. Moises Mallo.
All the animal work was conducted in compliance with Portuguese and European laws and was performed at the IGC animal house, licensed by the Direcção Geral de Veterinária (Laboratory permission 520/000/000/2518/99 and Portaria 1005/92 and Directive 86/609/EEC, respectively) following the FELASA recomendations.

3.2 Gene amplification and Plasmid construction

The M2 gene was amplified by PCR using Pfu DNA polymerase, from template DNA of MHV-68. The primers used were the following: Up 5'-atgaggtttcgttttcagg-3', Low 5'-ttactcctcgccccactccac-3'. PCR was conducted on a PTC-100 Peltier-Effect Cycling apparatus and conditions were dNTPs 200M, primers 1M, MgSO4 3mM, 95º-2', 30X (95º-1', 44-1', 74º-2'), 74º-5'. The gene was cloned into pMSCVneo, subcloned as a 5' EcoRII -3' XhoI fragment into the pCS2+ vector, and then subcloned with a downstream SV40 poly A tail as a 5' Cla I -3' Apa I fragment into the ps vector. This vector, constructed by Joanna B. Wilson, and provided by Dr. Pedro Simas (who cloned the M2 gene in the pS plasmid), is based on the plasmid backbone of pBluescript (SK) and incorporates the promoter (150bp) and enhancer (700bp) sequences of the mouse Ig heavy chain, upstream of the mouse APRT intron (150bp). The recombinant vector was digested with KpnI and SacI to release the expression cassette from the bacterial backbone vector. After being purified from an agarose gel, the expression cassette was microinjected into FVB/N fertilized eggs. These were then transferred into pseudo-pregnant foster females, producing the founder mice. These founders were crossed with wild-type FVB/N mice to obtain hemizygous mice. Subsequent crossing of hemizygous mice yielded homozygous animals. Control animals were littermates without the transgene.

3.3 Sequencing

The cloned M2 gene was sequenced using primers derived from the sequence of the ps plasmid upstream and downstream of the ClaI and XhoI sites, respectively: Up 5'-ctgcccggccacacgcgggtcactctc-3' and

Low 5'-cgatatcaagcttatcgataccgtcgacc-3'. The kit used was BigDye terminator v1.1, Part Nº 4336776, Applied Biosystems and the cycling conditions were 96º-1' and 25X (96º-10'', 50º-5'', 60º-4'). The PCR products were analysed on a 377 DNA Sequencer and 3130xl Genetic Analyser, Applied Biosystems.

3.4 Mouse genotyping by Southern Blot and PCR

Founder mice were analysed by Southern blot to assess integrity of the expression cassette injected into the fertilized eggs and discriminate between positive and negative mice. Genomic DNA (10g) isolated from tail biopsies was digested with KpnI and SacI and electrophoresed on a 0.8% agarose gel. Tails were digested overnight with Proteinase K (100g/ml) at 56ºC and then DNA was precipitated with isopropanol (0.7 volumes) at room temperature. Treatment of the gel, transfer to the membrane Hybond-N+ and hybridization were done according to the instructions of Hybond-N+ (Amersham, U.K.). The M2 probe was labelled with 32P-dCTP using the Random Primers DNA Labelling System, Gibco, according to the manufacturer's instructions. Unincorporated probe was removed using G-50 Sephadex Quick Spin Columns, Gibco.

Mice were then genotyped by PCR using DNA from tail biopsies obtained at the time of weaning (3 week-old). Detection of the M2 gene in the DNA by PCR was performed using Taq DNA Polymerase and the previously described primers. PCR was conducted on a PTC-100 Peltier-Effect Cycling apparatus and conditions were dNTPs 200M, primers 1M, MgCl2 2mM, 95º-2', 30X (95º-1', 54-1', 72º-2'), 72º-5'.

3.5 Determination of transgene copy number by real time quantitative RT-PCR (qRT-PCR)

The amount of amplified M2 transgene estimated by LightCycler Fast Start DNA Master SYBR Green I (Roche) (primers Up, 5'-atgaggtttcgttttcagg-3, Low, 5'-ttactcctcgccccactcc-3') was normalized against the amount of amplified mouse β-Globin (primers Up, 5'-ccaatctgctcacacaggatagag-3', Low, 5'-ccttgaggctgtccaagtgattca-3'). Conditions were $MgCl_2$ 4mM, primers 200nM. Program for M2 was: pre-incubation 95º-10', amplification 45X (95º-10', 70º-5'', 72º-30''), Melting Curve Analysis (65º-15''); program for β-Globin: pre-incubation 95º-10', amplification 45X (95º-10', 70º-5'', 72º-20''), Melting Curve Analysis (65º-15''). Analysis with the Roche supplied software and calculations on the amount of amplified product were based on the determination of the second derivative maximum and on an arithmetic adjustment of the baseline.

3.6 Demonstration of transgene expression by qRT-PCR

Total RNA was extracted from tissue homogenates of thymus, spleen and lymph nodes from transgenic and control littermates using Trizol Reagent, Sigma. Samples of RNA were digested with DNase I (Invitrogen) and cDNA synthesis was performed with MMLV-Reverse Transcriptase (Invitrogen), according to the manufacturers's instructions. Detection of the M2 gene in the cDNA by PCR was performed by RT-PCR using LightCycler Fast Start DNA Master SYBR Green I (Roche) as described above.

3.7 Flow cytometry analysis of lymphocyte surface proteins

Cell suspensions of bone marrow and spleen were incubated with titrated concentrations of the antibodies used in the staining combinations given in the text, washed in Phosphate Buffered Saline (PBS)-2% Foetal Calf Serum (FCS)-0.02% sodium azide and subsequently analyzed in a FACScalibur (Becton Dickinson, Palo Alto). The following rat anti-mouse monoclonal antibodies (BD Pharmingen, San Diego, CA) were used: Anti-IgM-Alexa488, anti-B220-APC, anti-CD23-PE, anti-CD21-FITC and anti-IgD-PE. Except for the four colour staining, propidium iodide (PI) was always used to exclude dead cells from the analysis. Data were processed using CellQuest software (Becton Dickson Immunocytometry Systems, San Jose, CA). Live lymphocyte counts were deduced from the acquisition of a fixed number of 10-µm latex beads (Coulter) mixed with a known volume of unstained cell suspension.

3.8 Stimulation and measurement of germinal centre formation

Mice were injected intraperitoneally with 200µl of 2% sheep red blood cells, and 7 to 10 days later spleens were collected, frozen in tissue-tek O.C.T. compound (Sakura Finetechnical), and 8µm cryostat sections were fixed (absolute ethanol, 5'-RT, followed by acetone, 5'-RT) and air-dried. Quantification of the germinal centres was performed by immunofluorescence using a titrated concentration of rat anti-mouse Ig coupled to Alexa 488 (BD Pharmingen, San Diego, CA) counterstained with biotinylated Peanut Agglutinin (PNA) (Vector Laboratories) followed by streptavidin-TexasRed (BD Pharmingen, San Diego, CA).

3.9 Immunizations with T-dependent (TD) antigens and Ig isotype-specific ELISA

Mice aged 16 to 20 weeks were injected intraperitoneally with DNP-KLH (TD) (100µg) (Biosearch Technologies) in PBS on day 0, re-challenged on day 21 and sacrificed on day 28. Blood was collected for serum on days 0, 7, 14, 21 and 28. Determination of DNP-specific serum antibodies was done by Ig isotype-specific ELISA. Flat-bottomed 96-well ELISA plates (Nunc Maxisorp, Naperville, IL) were coated with DNP-bovine serum albumin (BSA) (Biosearch Technologies) for 1h at 37ºC at a concentration of 5 µg/ml in K_2HPO_4 0.5M, KH_2PO_4 0.5M, pH=8.0. After washing three times with PBS (no $Ca2^+$ or $Mg2^+$) containing 0.1% Tween-20, blocking was performed at 37°C for 1h using 200µl/well of PBS containing 1% gelatine (PBS-G).
Serial dilutions of serum samples in PBS-G were added to the plates and left O/N at 4ºC. Plates were washed 5 times and bound isotype -specific antibodies were detected with biotinylated subclass-specific horseradish peroxidase (HRP) conjugated antibody (Southern Biotechnology Associates, Birmingham, AL), incubating at 37°C for 1h. The plates were washed 5 times and developed with freshly prepared 0,5mg/ml orto-phenylenediamine (OPD), 0.03% H_2O_2 in Na_2HPO_4 0.2M, Citric acid ($H_8C_6O_7$) 0.1M, pH=5.6 at RT and protected from light. The enzymatic chromogenic reaction was stopped with H_2SO_4 0.1 M and A492nm determined.
The initial dilution of the serum varied according to the Ig isotype and was empirically determined. The relative concentrations of DNP-specific antibodies are shown as A492nm values and for each isotype a single dilution factor that fell in the linear part of the curve is presented for all time-points (1:6400 for IgM, 1:3200 for IgG1, 1:1600 for IgG2a and IgG2b and 1:400 for IgG3).

3.10 Statistics

Statistical significance between the groups was determined by a two-tailed Mann-Whitney t test and no correction was made for multiple comparisons. Data were analysed using GraphPad Prism v5.0 statistical software. Statistical significance was accepted at a two-tailed P of <0.05 (*).

4 Results

4.1 Construction of transgenic mice, determination of the number of copies and expression of the M2 transgene

Selective B lymphocyte transgenic M2 expression in FVB/N mice was obtained from a litter of nine mice, after injection of fertilized eggs with the expression cassette of the pμs plasmid containing the M2 transgene. Southern blot analysis revealed two females and one male with a transgenic DNA fragment of the expected size. The two female founders were crossed with wild-type FVB/N male mice. The resulting F1 mice were further crossed to yield the F2 generation. Southern blot analysis of both founder mice of the M2 B cell restricted transgenic line revealed the presence of the injected expression cassette of the plasmid pμs (data not shown). The number of copies of the transgene incorporated in the genome of the two founders, as assessed by the Light Cycler technology, varied between 10 and 30. In all of the F1 and F2 mice bred from the two selected founders that were analyzed, expression was confirmed by RT-PCR **(Figure 1)**. The amplification of β-globin was used as a control for the quality of the RNA in both of the two founder lines. Besides that, protein expression was confirmed by fluorescent staining of spleen cells from SRBC immunized M2-transgenic mice **(Figure S1)**.

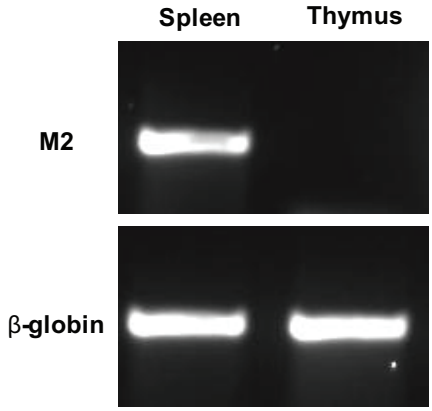


Figure 1. Seletive B lymphocyte expression of the M2 transgenic protein.
RT-PCR for the M2 gene in transgenic splenic (positive) and thymocyte (negative) cell suspensions. β-Globin loading controls in splenocytes and thymocytes below. The results are representative of at least 5 mice per founder.

4.2 B cell-specific expression of M2 does not affect subpopulations of B cells

To assess the role of M2 during B cell development, flow cytometric analysis of lymphocyte suspensions prepared from bone marrow and spleen were performed.

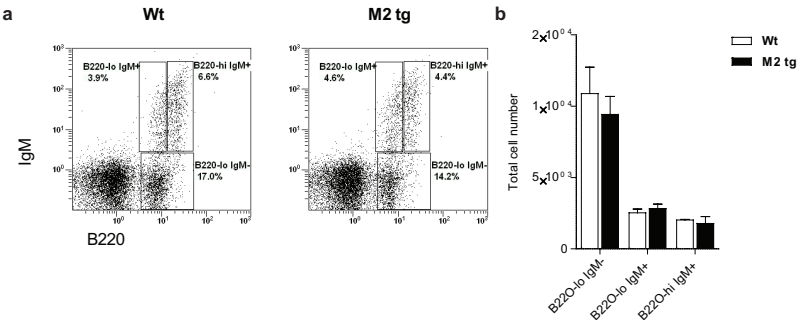


Figure 2. FACS analysis of B cells in control and M2 B cell restricted transgenic mice in bone marrow.
Cells were stained with B220 and IgM. The analysis was performed gating on B-lymphocytes and after exclusion of dead cells by incorporation of PI. The white and black bars represent the control and M2-transgenic mice, respectively. Representative FACS profiles **(2a.)** and the relative numbers of B cell subpopulations **(2b.)** are presented. The results (n=5, mean ± s. d) are representative of at least three independent experiments (P>0.05, not significant).

The frequency of bone marrow B-cell subpopulations, which define successive stages of B-cell development, was determined on the basis of IgM and IgD surface markers on B220+ cells pro or pre B cells $B220^{low}$ IgM^{-}, immature B-cells, $B220^{low}IgM^{+}$ and mature B cells $B220^{hi}IgM^{+}$ **(Figure 2)**.
The IgM: IgD ratios were similar in both control and transgenic mice immature B-cells, $IgM^{+}IgD^{-}$, transitional B cells $IgM^{+}IgD^{low}$ and mature recirculating B-cells, $IgM^{+}IgD^{+}$ **(Figure 3)**. The IgM:IgD ratios were similar in both control and transgenic mice **(Figure 3)**.

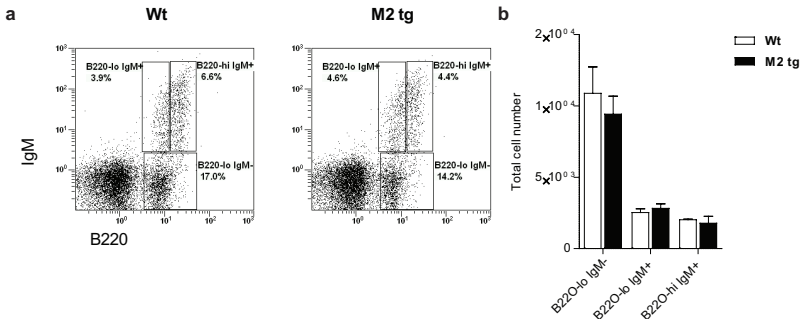


Figure 3. FACS analysis of B cells in control and M2 B cell restricted transgenic mice in the bone marrow.
Cells were stained with IgM and IgD. The analysis was performed gating on B-lymphocytes and after exclusion of dead cells by incorporation of PI. The FACS profiles are presented **(3a.)**. Relative numbers of B cell subpopulations. The white and black bars represent the control and M2-transgenic mice, respectively. **(3b.)**. The results are for groups of 5 mice per genotype and are representative of at least three independent experiments. P>0.05 (not significant).

Similarly, there was no difference between control and transgenic mice in Marginal Zone ($B220^{+}CD23^{-}CD21^{bright}$) and Follicular B cell ($B220^{+}CD23^{+}CD21^{+}$) subsets in the spleen **(Figure 4)**.
Collectively, these findings indicate that expression of M2 did not affect B-cell bone marrow development and splenic maturation. Similar results were also observed in the lymph nodes (results not shown).

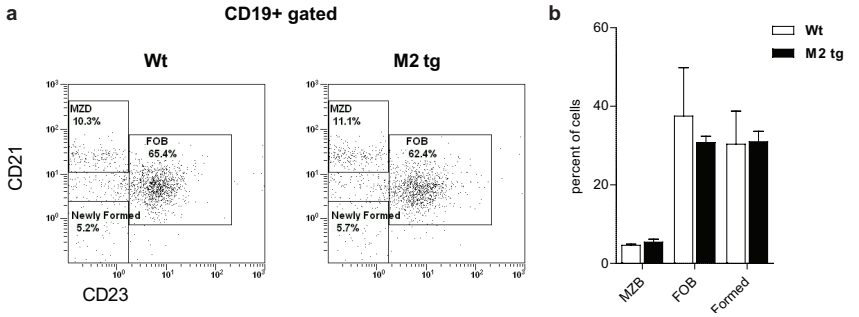
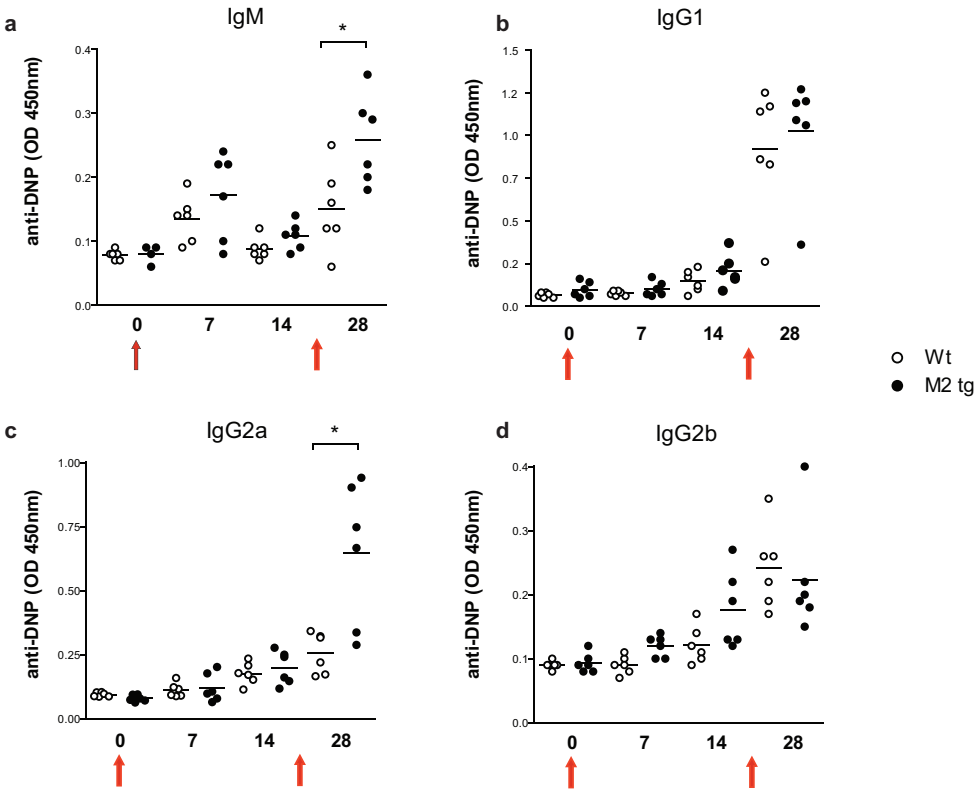


Figure 4. FACS analysis of B cells in control and M2 B cell restricted transgenic mice in the splenocytes.
Cells were stained with B220, CD21 and CD23 antibodies. The analysis was performed gating on B-lymphocytes and after exclusion of dead cells by incorporation of PI **(4a.).** Pooled data **(4b.).** The white and black bars represent the control and M2-transgenic mice, respectively. The FACS profiles are presented on the top and the relative numbers of B cell subpopulations on the bottom. The results are for groups of 5 mice per genotype and are representative of at least three independent experiments. P>0.05 (not significant).

4.3 M2 expression in B cells induces higher levels of secondary IgM and IgG2a antibody and increased numbers of germinal centres in response to T-dependent (TD)

To determine if the B cell restricted M2-transgenic mice can mount antigen -specific antibody responses, M2-transgenic and control wild-type mice were immunized with T-independent (DNP-LPS and DNP-Ficoll) and T-dependent (DNP-KLH) antigens. Similar anti-DNP antibody responses to DNP-LPS and DNP-Ficoll were observed in control and transgenic mice 10 and 21 days post immunization (data not shown). Levels of DNP-specific IgG1, IgG2a, IgG2b, IgG3, and IgM antibodies were measured 10 days after primary immunization and 7 days after the secondary immunization, (day 21) with DNP-KLH. There was no significant difference between control and transgenic titres of DNP-specific IgG1 and IgG2b antibodies, whereas titres of DNP-specific IgM and IgG2a were significantly elevated (*P<0.05) in the secondary response of the M2-transgenic mice at day 28. The higher levels of these antibodies to the T-dependent antigen **(Figure 5a and 5c)** are in agreement with the increased numbers of germinal centres determined by the number of peanut agglutinin (PNA) positive areas in the spleen sections, which reinforces the idea of an increased expansion of B cells during the germinal centre reaction in the M2-transgenic mice.


a
IgM
anti-DNP (OD 450nm)
0.4
0.3
0.2
0.1
0.0
*
0
7
14
28
b
IgG1
anti-DNP (OD 450nm)
1.5
1.2
1.0
0.7
0.5
0.2
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7
14
28
Wt
M2 tg
c
IgG2a
anti-DNP (OD 450nm)
1.00
0.75
0.50
0.25
0.00
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14
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d
IgG2b
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28

123

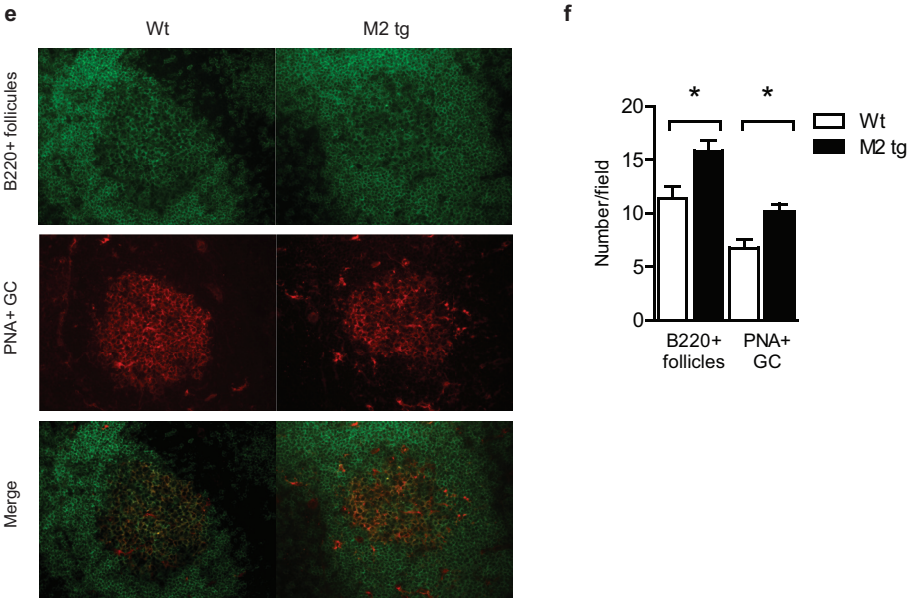
e
Wt
M2 tg
B220+ follicules
PNA+ GC
Merge
f
Number/field
20
15
10
5
0
*
*
Wt
M2 tg
B220+ follicles
PNA+ GC

Figure 5. Response of M2 B cell transgenic mice to TD antigen DNP-KLH and the formation of germinal centres in M2 B cell transgenic mice are increased.
Control and M2 B cell restricted transgenic mice were immunized with the TD antigen DNP-KLH. The relative amounts of DNP-specific IgM, IgG1 , IgG2a and IgG2b **(5a.-5d. respectivelly)** antibodies were determined by ELISA. Serum was collected before immunization and 7 and 14 days after primary immunization (day 1). Mice were then re-immunized at 21 days, bled at day 28 and the secondary immune response measured. The arrows represent the days at which mice were immunized (day 1 and day 21).
White and black circles represent mean ±S.D. for control and transgenic mice, respectively. Each circle represents one mouse. Results of the ELISA are shown as absorbance at 492nm (Y-axis) versus day of serum collection (X-axis). Similar results were observed in the lymph nodes (results not shown). Germinal Centre (GC) Formation in the M2 B cell restricted transgenic mice. Dual-colour immunofluorescence to visualize GC **(5e.)**. Numbers of splenic GC were calculated based on the numbers of B220+ follicular areas and PNA+ germinal centres in fields from histological cryostat sections from control and M2-transgenic mice (5f). Data are mean ± s.e.m. per microscopic field (18–20 fields were analyzed for each genotype). *P<0.05 in comparison indicated by horizontal line on chart.

5 Discussion

Virus host evasion strategies provide ready-made tools for genetic manipulation of cell biology and immune responses. The M2 gene of mouse herpes virus 68 (MHV-68) is known to be involved in the establishment, maintenance and reactivation of latency in B cells (Herskowitz *et al.*, 2005; Macrae *et al.*, 2003; Rodrigues *et al.*, 2006). Here, as an approach to better understand the biological role of M2, we have successfully constructed M2-transgenic mice with expression restricted to B cells. The construction of transgenic mice is a powerful alternative strategy to study mechanisms of immunity *in vivo*. The use of specific promoters to restrict cellular expression to a certain cell type is an additional refinement, which may overcome the undesirable effects of whole body transgenesis. Our findings reinforce the idea that the increased physiological expansion of B cells during the germinal centre reaction could increase the size of the memory B cell pool, the reservoir for virus *in vivo*. The data is consistent with previous works suggesting that M2 may manipulate the activation, proliferation and survival of B cells.

The validity of the model was justified by the generation of two founder lines with similar characteristics and with RT-PCR assays demonstrating expression of M2 in splenocytes but not in thymocytes. The first step in the characterization of the M2-transgenic mice was the analysis of surface markers. There were no differences between the wild-type and the M2-transgenic mice in their B cell subpopulations in the bone marrow, spleen and lymph nodes **(Figure 2-4)**. Therefore, the B cell restricted expression of the MHV-68 M2 protein *in vivo* did not impact on B cell development, a finding consistent with the absence of a strong *in vivo* phenotype for the VAV1, VAV2 and VAV3 triple knockout mice (Pearce *et al.*, 2004). As the downstream target for M2 is VAV 1/2, we may conclude that the VAV proteins are redundant and overlapping in their function.

We then performed functional assays to assess the impact of the transgene on the serological immune response. Transgenic mice immunized with the T- dependent antigen (DNP-KLH), produced statistically higher levels of IgM and IgG2a anti-DNP antibodies than normal mice **(Figure 5a-5d)**. The predominant subtype observed following MHV-68 infection, as well as in other viral infections, is IgG2a, suggesting a preferential class switching (Coutelier, 1987). Similarly, when M2-transgenic mice were challenged with a T-dependent antigen, there was a significant increase of IgG2a, especially in the secondary challenge.

Particularly significant was our observation of increased germinal centre formation in mice immunized with sheep red blood cells **(Figure 5e-5f).**

Together with the previous described data with M2 transfected WEHI-231 B cell lines *in vitro* (Madureira *et al.*, 2005), our results suggest that M2, perhaps through its interaction with Vav (Madureira *et al.*, 2005; Pires de Miranda *et al.*, 2008) increases the sensitivity of the latently infected B cells to activation via the immunoglobulin receptor (BCR), and thus might favour reactivation and consequent virus transmission. In addition, these experiments suggest that M2 might function to promote the survival of activated and memory B cells, thus expanding the reservoir of host cells for the establishment of latency.

Several studies with M2 deletion mutant viruses have shown reduced levels of acute splenic latency (Herskowitz *et al.*, 2005; Jacoby *et al.*, 2002; Macrae *et al.*, 2003), an effect attributed to a reduced number of infected splenic B cell follicles, rather than a reduced expansion of latently infected germinal centre B cells (Simas *et al.*, 2004). In addition, the M2 protein alone can drive plasma cell differentiation in a B lymphoma cell line (Liang *et al.*, 2009), perhaps providing a novel viral strategy to regulate gammaherpes virus reactivation from latently infected B cells. Thus, M2 has been postulated to be a new class of herpesvirus gene products (reactivation conditioners) that does not directly participate in virus replication, but rather facilitates virus reactivation by manipulating the cellular milieu to provide a reactivation competent environment (Liang *et al.*, 2009). Accordingly, a crucial role of M2 in the establishment of latency may be its impact in signalling the end of the expansion of the viral load in germinal centres with the associated differentiation of latently infected B cells into long-lived memory B cells, the main reservoir for the virus (Rodrigues *et al.*, 2006). In conclusion, M2 does not affect the distribution of B cell subsets in the bone marrow and spleens of the M2-transgenic mice but it affects B cell responses to thymus dependent protein antigens.

6 Acknowledgments

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Suplementary figure S1

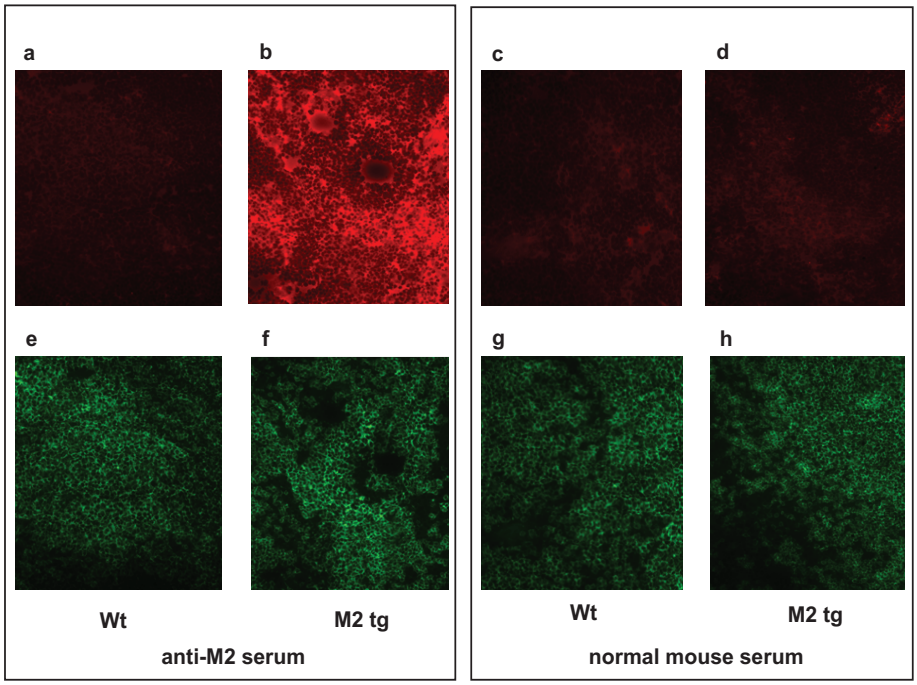

Figure S1. Expression of M2 by B-cells in spleen cell sections from SRBC immunized M2-transgenic mice.

Mice were immunized with SRBC and cryostat sections of spleen were fixed as described in Materials and Methods. Control **(S1a. and S1e.)** and transgenic **(S1b. and S1f.)** spleen sections were stained with a mouse-anti-recombinant M2 serum followed by biotin anti -mouse IgG2a (Biolegend) and streptavidin-Texas Red (Southern Biotechnology) **(S1a. and S1b.)** and counterstained with FITC-monoclonal-anti-mouse B220 (Ebiscience) **(S1e and S1f)**. As a specificity control for the anti-M2 staining, spleen sections were stained with normal mouse serum followed by biotin anti-mouse IgG2a (Biolegend) and streptavidin-Texas Red (Southern Biotechnology) **(S1c. and S1d.)** and counterstained with FITC-monoclonal-anti-mouse B220 (Ebiscience) **(S1g. and S1h.).**

Note the low background in the staining with the biotin anti-mouse IgG2a (Biolegend) and streptavidin-Texas Red (Southern Biotechnology) **(S1c. and S1d.).** The recombinant M2 was expressed in E. coli transformed with a pGEX-M2 construct and purified by GST-affinity chromatography according to the manufacturer's instructions (GE Healthcare).

04 | Role of M2 in the Pathogenesis of MHV-68 in B-Lymphocyte Restricted M2-Transgenic Mice

1 Abstract

Some important features of M2 protein in the murine herpesvirus strain-68 (MHV-68) pathogenesis were already indicated by *in vitro* and *in vivo* analysis by using M2 mutant virus, but the precise function of M2 and its specific role in MHV-68 latency remain to be clarified. In order to investigate the immunological and virological aspects of this specific protein in the MHV-68 pathogenesis, we combine the use of our M2 B-cell restricted transgenic mice with the use of MHV-68 knockout to ORF M2 viruses. This approach provides an alternative system model to explore the impact *in vivo* of a single virus protein in the context of the latent phase of infection,

Here we show that the pathogenesis of M2-transgenic mice infected with the M2-deficient mutant virus did not revert to that observed upon infection of normal mice with Wt virus. However, the higher reactivation levels late after M2-transgenic mice were infected with Wt virus reflected the importance of M2 as a target for the immune response, and thus with an impact on the establishment of latency. Finally, there was markedly less apoptosis in B cells from M2-transgenic mice infected with either Wt or M2FS mutant, than from similarly infected wild-type mice, coherent with the published inhibitory influence of M2 on apoptosis *in vitro*. Thus M2 plays a role in both the humoral and cellular anti-virus immune response and provides a strategy to increase the pool of germinal centre B cells through inhibition of apoptosis in the infected cell.

2 Introduction

The murine herpes virus (MHV)-68-infected mice are well known as a model for studies of gammaherpesvirus infection. Intranasal inoculation of mice with MHV-68 results in a productive infection in the lungs, with maximal viral titres observed between days 5 and 10-post infection (p.i.), and viral clearance on days 9-15 post infection. During the subsequent viremia, MHV-68 spreads to the spleen where it becomes latent, mainly in B lymphocytes located in the germinal centre and in the memory B lymphocyte pool (Flaño *et al.*, 2003; Thorley-Lawson & Babcock, 1999; Thorley-Lawson, 2001; Willer & Speck, 2003). B cells are required for trafficking of infected cells to the spleen, leading to the establishment of splenic latency (Stewart *et al.*, 1998; Weck *et al.*, 1999). During latent infection, there is no virus replication and the viral genome is present as a nuclear episome (Clambey *et al.*, 2002). Initial establishment of latency in the spleen is associated with a marked splenomegaly and mononucleosis. The splenomegaly is driven by CD4+ T cells and is dependent on the presence of MHV-68-infected B cells in the spleen (Usherwood *et al.*, 1996). The resolution of splenomegaly is achieved by CD8+ T cells, which are also important in the long-term control of persistent infection (Ehtisham *et al.*, 1993).

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This work focuses on the role of the M2 gene; a gene transcribed during latency, with a pivotal role in the establishment, maintenance and reactivation from latency in B cells (Herskowitz *et al.*, 2005; Jacoby *et al.*, 2002; Macrae *et al.*, 2003) and a major target for the host cytotoxic T lymphocyte response (Husain *et al.*, 1999). Studies with M2-deficient viruses indicate that M2 does not have an impact on the acute phase in the lung (Jacoby *et al.*, 2002; Macrae *et al.*, 2003).

Several studies with M2 deletion mutant viruses have shown reduced levels of acute splenic latency (Herskowitz *et al.*, 2005; Jacoby *et al.*, 2002; Macrae *et al.*, 2003), an effect attributed to a reduced number of infected splenic B cell follicles, rather than a reduced expansion of latently infected germinal centre B cells (Simas *et al.*, 2004). In addition, the M2 protein alone can drive plasma cell differentiation in a B lymphoma cell line (Liang *et al.*, 2009), perhaps providing a novel viral strategy to regulate gammaherpes virus reactivation from latently infected B cells. Thus, M2 has been postulated to be a new class of herpesvirus gene product (reactivation conditioners) that does not directly participate in virus replication, but rather facilitates virus reactivation by manipulating the cellular milieu to provide a reactivation competent environment (Liang *et al.*, 2009). Accordingly, a crucial role of M2 in the establishment of latency may be its impact in signaling the end of the expansion of the viral load in germinal centres with the associated differentiation of latently infected B cells into long-lived

memory B cells, the main reservoir for the virus (Rodrigues *et al.*, 2006). M2 has been shown to interact with a number of cellular proteins *in vitro*. M2 co-immunoprecipitates with Vav1 and Vav2 proteins in 293T and inhibits cell cycle arrest and apoptosis in WEHI-2931 B-cells (Madureira *et al.*, 2005). In fibroblast cultures, M2 interacts with DDB1/COP9/cullin repair complex and ATM to suppress DNA-damage induced apoptosis (Liang *et al.*, 2006). However, to date the impact of M2 expression *in vivo* has not been reported. Thus the main goal of this part of the work was to assess the *in vivo* role of M2 in the host response to MHV-68 virus infection during the latency state, using the B-cell restricted M2-transgenic mouse.

3 Materials and methods

3.1 Cell culture

BHK-21 cells were cultured in 5% CO_2 at 37°C in complete GMEM, (cGMEM) consisting of Glasgow's modified Eagle's medium (GMEM) supplemented with 10% FCS, 10% (w/v) tryptose phosphate broth, 100U/ml penicillin-G, 100µg/ml streptomycin and 2mM L-glutamine.

3.2 Virus production and titration

MHV-68 clone G2.4 used (Efstathiou *et al.*, 1990) in this work was provided by Dr. Pedro Simas. The MHV-68 recombinant virus (M2FS) (provided by Dr. Heiko Adler and Dr. Stacey Efstashiou) contains a frameshift in the ORF M2 created by insertion of a single guanine between nucleotides 4603-4604, immediately downstream of the translational start codon (Husain *et al.*, 1999), creating a novel Apa I site and premature stop codon 78 nucleotides downstream. Virus working stocks were grown by low multiplicity infection of semi-confluent BHK-21 cells (0.001 PFU/cell) in 150cm^2 culture flasks, with MHV-68 or with M2FS. After 4 days, supernatants were centrifuged (30000*g*, 2h, 4°C, Beckman type 19 rotor) and pelleted virus was re-suspended in 2ml GMEM medium and ultrasonically disrupted for 30sec. Aliquots were stored at -80°C. To determine the virus titre sub-confluent BHK-21 cells were incubated with 10-fold serial dilutions of virus in GMEM. Following a 1h adsorption, the medium was replaced with cGMEM medium containing 0.32% carboxymethyl cellulose and incubated at 37°C for 4 days. After removal of this medium, cells were fixed with 10% formaldehyde in PBS and then stained with 0.1% toluidine blue in formal saline. Viral plaques were counted under a Stermi SV6 microscope (Zeiss) and virus titres were calculated from numbers in duplicate dishes.

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3.3 Infection of mice with MHV-68 or M2FS

After genotyping, 6-8 week old mice were transferred from the standard animal house to quarantine. Animals were inoculated intranasally under the effect of light halothane anaesthesia with 10^4 PFU of MHV-68 or M2FS virus in 20µl of PBS. At different time points after infection, mice were killed by inhalation of CO_2. The entire experiment was performed twice, with comparable results.

3.4 Infectious centre assay

Spleen cell suspensions were 10-fold serially diluted, starting with 10^7cells/ml. BHK-21 cells ($5x10^5$) were added to each sample and the final volume was adjusted to 5ml with cGMEM. The mixture incubated for 1h at 37°C with gentle shaking and then plated out in 60mm cell culture Petri dishes, was then incubated at 37°C in a humidified incubator for 5 days. Monolayers were fixed with 10% formaldehyde in PBS and stained with 0.1% (w/v) toluidine blue in formal saline. Plaques were counted under a Stemi SV6 microscope (Zeiss) and the number of infectious centres per 10^8 splenocytes was calculated.

3.5 Estimation of apoptotic cells

Splenic cell suspensions were stained with a titrated concentration of a monoclonal FITC labeled rat anti-mouse B220 (BD Pharmingen, San Diego, CA) to allow B cell selection. Then, these suspensions were fixed in 90% ethanol (20' at RT), washed with PBS, permeabilized (PBS containing 0.05% (v/v) triton X-100 and 50 units of RNaseA) for 30' at RT. After this, samples were re-suspended in PBS with propidium iodide (PI), at a final concentration of 25μg/ml and analysed in a FACScalibur (Becton Dickinson, Palo Alto). Apoptotic B cells were determined accounting cells in the hypodiploid sub-G1 peak after gating on the B220+ splenic B cell population.

3.6 Statistics

Statistical significance between the groups was determined by a two-tailed Mann-Whitney t test and no correction was made for multiple comparisons. Data were analysed using GraphPad Prism v5.0 or SPSS v17.0 statistical software. Statistical significance was accepted at a two-tailed P of <0.05 (*).

4 Results

4.1 Pathogenesis of normal and B cell restricted M2-transgenic mice to infection with MHV-68 and M2 deficient (M2FS) virus

To study the pathogenesis of MHV-68 infection in the M2-transgenic mice, groups of M2-transgenic and wild-type mice were infected intranasally with 10^4 PFU of MHV-68 or M2FS viruses and monitored by infectious centre assays over a period of 90-days post infection (p.i.) to determine levels of latent viruses in the spleen.

First, comparing wild-type FVB/N mice infected with MHV-68 or M2FS virus, at day 14 p.i. there was an approximately 10-fold reduction in the number of infectious centres in mice infected with M2FS virus, as compared with mice infected with MHV-68, which is in agreement with similar work in BALB/c mice (Simas *et al.*, 2004) **(Figure 1a)**. At later time-points, days 43 p.i. **(Figure 1c)** and 71 p.i. **(Figure 1d)**, the number of infectious centres was consistently higher in the M2FS virus infected mice, when compared with mice infected with wild-type MHV-68, as previously described in BALB/c mice (Marques *et al.*, 2008; Simas *et al.*, 2004). Importantly, even at 90 days p.i. **(Figure 1e)**, the long-term latency of MHV-68 in FVB/N mice was not significantly affected by the disruption of the M2 protein, also as previously described for BALB/c mice. Thus, we confirm that the acute latency deficit and long-term latency of M2 deficient MHV-68 virus observed in BALB/c mice (Simas *et al.*, 2004) also occurs in FVB/N mice.

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Second, comparing infection of M2-transgenic and wild-type mice infected with MHV-68 virus, reactivation levels were significantly higher at later times [day 43 **(Figure 1c)** and day 90 **(Figure 1e)**] in M2-transgenic mice infected with wild-type virus, consistent with the proven importance of M2 as a target for the protective immune response and thus with an impact on the establishment of latency.

Third, comparing infection of wild-type and M2-transgenic mice with M2FS there were no major differences between the FVB/N wild-type and the M2-transgenic mice, apart from day 71 p.i.; where a higher number of latent reactivation-competent virus was observed in the wild-type mice infected with M2FS mutant virus **(Figure 1d)**.

Fourth, and finally, comparing wild-type and M2-transgenic mice infected with MHV-68 and the M2FS mutant virus,

infection of the M2-transgenic mice with the M2-deficient virus (M2FS) did not result in a restoration of the latency deficiency seen in wild-type mice infected with the M2FS virus. Thus, at day 43 p.i. similar numbers of infectious centres were observed, and therefore a similar latency deficiency, recovered from both wild-type and M2-transgenic mice infected with the M2 deficient virus **(Figure 1c)**.

Surprisingly, the latency deficiency observed upon infection of normal mice with the M2 deficient virus was not reversed upon infection of M2-transgenic mice with the M2 deficient virus **(Figure 1a)**. An explanation for this is not immediately obvious, but one possibility could be the impact of the M2 transgene on the physiology of the B cell. For example, it is known that M2 drives B cell proliferation and differentiation in an IL-10 dependent manner (Siegel *et al.*, 2008). For this reason, we measured the IL-10 levels in sera from naïve and SRBC stimulated normal and M2-transgenic mice. Although the levels of IL-10 were low, they were, nevertheless, significantly above background ($p<0.05$), and there was no significant difference between the wild-type and M2-transgenic mice ($p>0.05$, not significant) **(Figure S2)**.

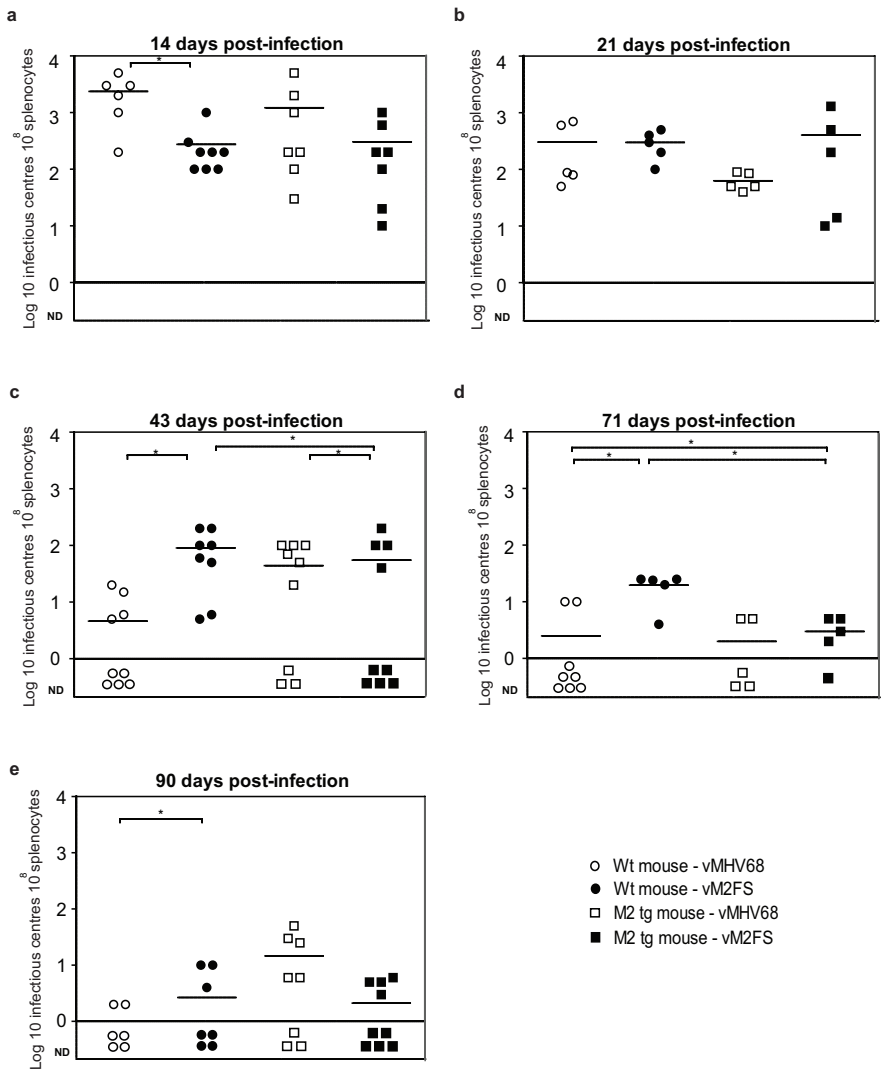

Figure 1. Numbers of latent reactivation-competent virus in control and M2-transgenic mice infected with MHV-68 or M2FS virus.

Control and M2-transgenic mice were infected intranasally with 10^4 PFU of either MHV-68 or M2FS and the spleens were assayed for latent virus at the days from 14 to 90 days post-infection (p.i.). Wild-type mouse with MHV-68 virus (white circles), Wild-type mouse with M2FS virus (black circles), M2-transgenic infected with MHV-68 virus (white circles), or M2-transgenic infected with M2FS virus (black circles). Results were compiled from two independent experiments, and the results for these four groups are presented as the numbers of latent reactivation-competent virus measured on days 14, 21, 43 , 71 and 90 **(1a. to 1e., respectivelly)** post-infection. The continuous horizontal dashed line indicates the limit of detection of the assay and the short horizontal line in each group of circles represents the arithmetic mean (ND, denotes that virus was not detectable). Each circle represents an individual mouse. *P<0.05 in comparison indicated by horizontal line on chart.

4.2 Expression of the M2 protein protects against apoptosis induced by infection with MHV-68

To study the impact of the M2 transgene in the context of infection, groups of 8 week old wild-type and M2-transgenic mice were infected intranasally with wild-type (MHV-68) and M2 deficient recombinant (M2FS) viruses. Spleen cell suspensions prepared 21 days post-infection were stained with Propidium Iodide (PI) and apoptosis was assessed by the magnitude of the hypodiploid sub-G1 peak. In contrast to the abundant apoptosis in the normal mice infected with either of the two viruses, (Wt 38,3±11% and M2FS 49,2±4,32%), there was little or no apoptosis with either virus, (Wt 0,08±0,08% and M2FS 0,00±0,05%) in the B-cell from M2-transgenic mice **(Figure 2a and 2b)**, a finding consistent with observations *in vitro* (Madureira *et al.*, 2005).

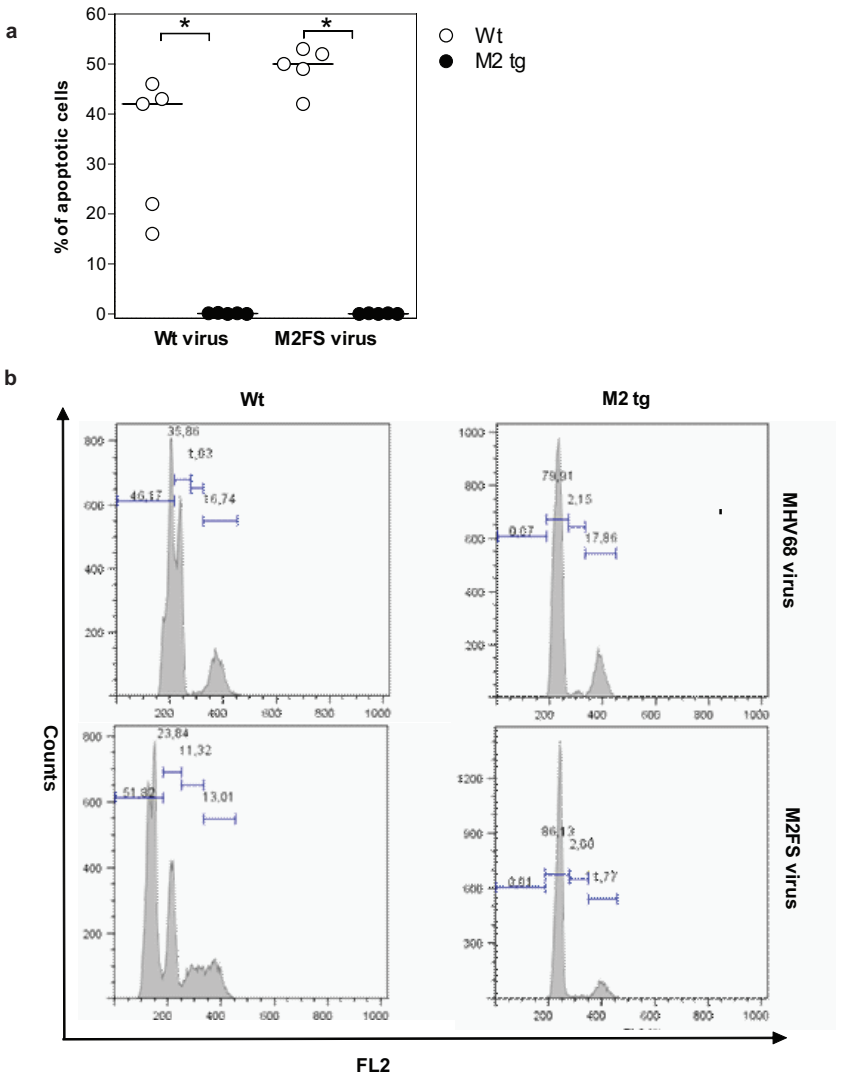

Figure 2. Transgenic expression of the M2 transgene protects B-cells from apoptosis.

Suspensions of splenocytes from control (left hand side) and M2-transgenic (right hand side) mice infected with 10^4 PFU of either MHV-68 or M2FS virus were stained with a FITC monoclonal rat anti-B220 to identify the B cells, permeabilized, stained with Propidium Iodide (PI) and examined by FACS. Apoptotic cells were determined by the presence of a hypodiploid sub-G1 peak after gating on the B220+ splenic B-cell population. **2a.** Results are presented as the percentage (%) of apoptotic cells for the control (white circles) and the M2-transgenic mice (black circles). Each circle represents an individual mouse. **2b.** Representative FACS profiles are also shown.

The results are for groups of 5 mice per genotype and are representative of at least three independent experiments. *P<0.05 in comparison indicated by horizontal line on chart.

5 Discussion

The M2 gene of mouse herpes virus 68 (MHV-68) is known to be involved in the establishment, maintenance and reactivation of latency in B cells. The work presented here complements the work presented in Chapter 3 challenging the M2 B-cell restrict transgenic mice with wild-type and mutant virus infections to further clarify the role of M2 in host-virus interaction.

Previous studies, conducted with female BALB/c mice, demonstrate that recombinant viruses, deficient in M2, show no replication deficit, neither in tissue culture nor during the acute phase in the lungs of infected mice (Jacoby *et al.*, 2002; Macrae *et al.*, 2001). Thus, before investigating the pattern of establishment and maintenance of latency in the FVB/N M2-transgenic mice infected with MHV-68 and M2FS deficient viruses, we analyzed the infection in the wild-type FVB/N background. Indeed, we observed that FVB/N wild-type mice infected with M2FS revealed the same phenotype as BALB/c, namely, an acute latency deficit and an elevated long-term latency. Therefore control of infection through recognition of M2 peptides by CD8+ T-cells might not be restricted to the H-2K^b haplotype, as previously suggested (Marques *et al.*, 2008).

In order to explore the role of M2 in the establishment of latency M2-transgenic and wild-type mice were infected with wild-type MHV-68 and M2 deletion mutant (M2FS) viruses. Then the numbers of reactivation-competent virus plaques in the spleen were measured as an indication of latently infected B-cells **(Figure 1a-1e)**. The yield of virus recovered in normal mice infected with the M2 deficient virus increased at the late, but not at earlier phase of infection. This phenotype was not observed in the M2-transgenic mouse, where the response to wild-type and M2 deficient viruses was similar. The observation of higher reactivation centres in the late phase of latency in the M2-transgenic mice infected with wild-type viruses is consistent with the role of M2 in expansion of germinal centre B cells, on the one hand. On the other hand, the higher virus yield in M2-transgenic mice may be attributed to tolerance to M2 and the consequent loss of a major immunodominant CD8+ T-cell epitope with an expected impact on the establishment of persistence (Husain *et al.*, 1999).

In conclusion, the B-cell restricted transgenic expression of the MHV-68 ORF M2 provides a novel approach to explore the mechanism and possible exploitation of the M2 gene. It has the merit of being relevant in the context of an MHV-68 infection, providing a good platform to study the impact *in vivo* of a single virus protein during acute and latent phases of virus infection. Although we have not found an effect on the distribution of B-cell subsets in the bone marrow and spleens of the

M2-transgenic mice (described in chapter 3), we have found a relevant phenotype when these mice were challenged with a thymus dependent protein antigen, or when infected with MHV-68 or an M2 deficient recombinant virus. Infection of normal mice with M2 deficient MHV-68 resulted in an increased reactivation in the late, but not the earlier phase, of latency, an observation not seen when the M2-transgenic mice were similarly infected. More importantly, we have demonstrated *in vivo* that the host evasion M2 protein not only contains an immunodominant CD8+ T cell response, but also protects B-cells from apoptosis *in vivo*, thereby providing a viral strategy for increasing the pool of potential host cells for the establishment of latency.

In contrast to our expectation, the latency deficiency phenotype associated with the M2 deficient virus was not reversed by similar infection of M2-transgenic mice. The possibility that this might be related to the known impact of M2 on IL-10 expression (Siegel *et al.*, 2008) was not supported under our experimental conditions, as there was no significant difference between levels of serum IL-10 in control and SRBC immunized normal and M2-transgenic mice. Another possibility is that the impact of expression of the M2 transgene on the physiology of B cells might in some, as yet unexplained, way have been responsible for the failure to reverse the latency deficiency phenotype.

Our findings not only confirm the importance of the M2 protein as an immunodominant determinant of cellular immunity, but also shows that M2 is a key regulator of apoptosis in the germinal centre. Based on these results we suggest that M2 might function to promote the survival of activated and memory B cells, thus expanding the reservoir of host cells for the establishment of latency.

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Suplementary figure S2

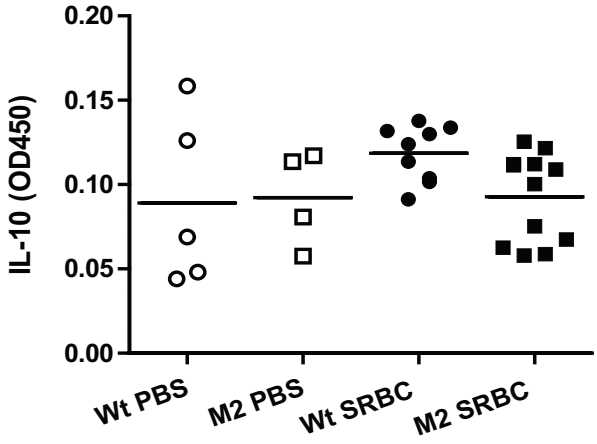

Figure S2. No significant difference of IL-10 levels in control and SRBC immunized normal and M2 transgenic FVB/N mice.
Mice were immunized as described in materials and methods and the sera were assayed for IL10 using murine IL-10 ELISA Development Kit (Peprotech) according to the manufacturer's instructions. The values are given for each mouse individually. P>0.05 (not significant).

05 | Final discussion

1 Viral evasion research

Coexistence of viruses and their hosts imposes an evolutionary pressure on both virus and the many defensive possibilities of the innate and adaptive host immune systems. On the one hand, the host has developed an extraordinarily versatile immune system able to recognize pathogens, extracellular and intracellular whereas pathogens on the other hand, viruses have reciprocally evolved a wide range of strategies to manipulate the host cell biology and immune system. Although the study of viral genes has been intensively explored, much more investigation is still required for a complete understanding of the role many various host evasion proteins in the complete scenario of host-pathogen interaction. In this context, our lab sees the study of virus-host evasion genes as a genuine source of ready-made tools for future therapeutics. Therefore our focus is first to identify and then exploit host evasion mechanisms of assigned and non-assigned viral evasion genes, which the former can be identified through bioinformatics the latter can only be detected through functional assays. Previously, our lab demonstrated the importance of host-evasion mechanisms of some non-assigned viral evasion proteins from large DNA viruses, such as MHV-68, HCMV and AFSV, for example, the inhibition of cytokine and interferon responses (Correia, 2008) and the manipulation of cell cycle/apoptosis (Nascimento *et al.*, 2011). One in particular (A238L) was used for the construction of a T cell restricted “evasion gene” transgenic mouse to explore mechanisms operating in the development of the lymphoid system (Almeida *et al.*, 2012).

In this thesis, we have elucidated the mechanism of two other different viral immunomodulatory genes, ORF I329L (ASFV) and ORF M2 (MHV-68). These were identified through functional and bioinformatics analysis and their impact as transgenes was evaluated *in vitro* and *in vivo*, respectively.

1.1 African Swine Fever virus gene I329L, a TLR3 antagonist *in vitro*

In the second chapter of this thesis, we have systematically analysed an unassigned gene from African SwineFever Virus, ORF I329L, characterizing its biological role as an inhibitor of TLR3 signalling. This research was motivated by the hypothesis that inhibition of TLR signalling may constitute an advantage for ASFV which must not only survive in both vertebrate and invertebrate hosts, but which is restricted to macrophage, the ‘motor’ of innate immunity, in both

hosts. We have identified through conventional bioinformatics a marginal sequence homology between I329L and TLR3. Subsequently, by biochemical analysis, we have characterized this unassigned gene as a highly glycosylated protein expressed in the cell membranes and on its surface, locations where many receptor and adaptor molecules involved in innate immunity have been identified (Fan *et al.*, 2008).

Significantly, TLR3 is described as one of the most heavily glycosylated TLRs (Bell *et al.*, 2006). Functional assays revealed that I329L inhibited dsRNA-stimulated activation of NFkB and IRF3, two key players in innate immunity. Consistent with this, expression of I329L protein also inhibited the activation of interferon-b and CCL5. Finally, overexpression of TRIF reversed I329L-mediated inhibition of both NFkB and IRF3 activation, suggesting that TRIF, a key MyD88-independent adaptor molecule, is a possible target of this viral host immunomodulation gene. Although this is the first ASFV gene manipulating TLRresponses to be described, the Vaccinia virus has evolved three proteins, A46R, A52R and N1L, that are inhibitors of the TIR-mediated immune response (Bowie & Unterholzner, 2008). Both A46R and A52R are able to inhibit signaling via multiple TLRs although the specificity varied showing that they are not redundant (Bowie *et al.*, 2000; Harte *et al.*, 2003). *In vivo* loss of either protein caused a mild attenuation in a murine intranasal model of infection. The third protein, N1L, is a small intracellular protein that contributes to virus virulence in a more profound manner (Bartlett *et al.*, 2002), targeting the I-kappaB kinase complex and inhibiting signalling via TLRs and tumor necrosis factor family of receptors (DiPerna *et al.*, 2004).

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Our early experimental results together with a bioinformatic approach, inspired a theoretical co-related work performed by Elsa Henriques in collaboration with our team (Henriques *et al.*, 2011). Using comparative structure modelling of the TLR3 and ORFI329L we hypothesized that I329L may function as a TLR3 decoy, showing that the viral protein could hinder TLR3 dimerization and inhibit the downstream signalling pathway. Our preliminary experimental data using FACS analysis of HEK-293T cells transfected with TLR3 and stimulated with Poly I:C indicated that I329L does not bind to dsRNA, but inhibits recognition of dsRNA by TLR3 intracellularly (unpublished data). Although our experimental data indicates that ORF-I329L-mediates intracellular inhibition through interaction with TRIF, the possibility that I329L protein interacts with the specific intracellular area responsible for TLR3 homodimerization, cannot be excluded. Based on our experimental data and the bioinformaticdata presented, some hypothetical models for the ASFV TLR-based strategies to evade the host defence by targeting TLR3 were considered.

1.2 B cell restricted transgenic expression of M2 MHV-68 protein *in vivo*

In the third and fourth chapter of this thesis, we have reported the successful construction a B cell restricted transgenic mouse, as an alternative approach to explore the mechanism and exploitation of a specific virus host evasion protein on a single cell level. Previous work from our team already validated this approach to study the impact of another viral evasion gene from African Swine Fever Virus, ORF A238L (Almeida *et al.*, 2012). An innovation of this work in particular, is that it allows the assessment of the biological effect of a specific virus transgene in association with the pathogenesis, during concomitant infection with the same virus, both wild-type and viral gene deficient, thus providing a good platform to study the impact *in vivo* of a single virus protein during acute and latent phases of virus infection. In this case, the selected M2 viral transgene is an already characterized viral host manipulation gene, implicated in the establishment, maintenance and reactivation of latency in B cells (Herskowitz *et al.*, 2005; Macrae *et al.*, 2003; Rodrigues *et al.*, 2006) .

Although we have not found an effect on the distribution of B cell subsets in the bone marrow and spleens of the B-cell restricted M2 transgenic mice, we have found an interesting phenotype, either when the transgenic mice were challenged with a thymus dependent protein antigen (DNP-KLH and SRBC), or when they were infected with MHV-68 or an M2 deficient recombinant virus. The latter findings are consistent with previous works describing M2 as a critical viral protein for B cells during the establishment and maintenance of latency in MHV-68 pathogenesis.

The predominant subtype observed following MHV-68 infection, as well as in other viral infections, is IgG2a, suggesting a preferential class switching (Coutelier, 1987). Interesting, in our hands, transgenic mice just immunized with the T-dependent antigen (DNP-KLH) also produced significant higher levels of IgG2a anti-DNP antibodies than normal mice, especially in the secondary challenge. This may be due to the increased germinal centre formation observed in M2 transgenic mice immunized with sheep red blood cells. It is possible that the increased germinal centre formation was the result of the significant lower levels of apoptosis in the virus infected transgenic splenic B-cells. In view of these findings it would be interesting to determine the affinity of the IgG2a antibodies in wild-type and M2-transgenic mice, the prediction being lower although (with less apoptosis) in the transgenics.

Infection of normal mice with M2 deficient MHV-68 resulted in an increased reactivation in the late, but not the earlier phase, of

latency, an observation not seen when the M2 transgenic mice were similarly infected. More interesting, the observation of higher reactivation centres in the late phase of latency in the M2 transgenic mice infected with wild-type viruses is consistent with the proven importance of M2 as a determinant of protective cellular immunity, and thus with an impact on the establishment of persistence (Husain *et al.*, 1999).

Together these data provide strong evidence that M2 manipulate the activation, proliferation and survival of B cells *in vivo*. These findings not only confirmed the importance of the M2 protein as an immunodominant determinant of cellular immunity, but also demonstrated *in vivo* that the M2 protein protects B cells from apoptosis *in vivo* validating previous described data with M2 transfected WEHI-231 B cell lines *in vitro* (Madureira *et al.*, 2005), thereby providing a viral strategy for increasing the pool of potential host cells for the establishment of latency. Thus M2 might function to promote the survival of activated and memory B cells, thereby expanding the reservoir of host cells for the establishment of latency. We hypothesized that M2, perhaps through its known interaction with Vav (Madureira *et al.*, 2005; Pires de Miranda *et al.*, 2008) increases the sensitivity of the latently infected B cells to activation via the immunoglobulin receptor (BCR), and thus might favour reactivation and consequent virus transmission.

2 Final remarks

The results of this work have contributed to the understanding of the impact of two different viral immunomodulatory proteins at the cell and whole animal level. At the cell level, we demonstrated in this thesis the major impact of I329L on innate immunity, which has never been described before. Besides adding to our understanding of the ASFV genes involved in immune evasion, these findings may contribute to the development of new and rational therapeutic approaches. The I329L gene may constitute a good candidate for development of therapeutics for autoimmunity and chronic inflammatory disorders or creation of an attenuated deletion ASFV mutant vaccine.At the animal level, we demonstrated an approach relevant to study the impact *in vivo* of a single virus protein during MHV-68 latency. Here, our data not only confirmed data that M2 is able to manipulate the activation and proliferation of B cells *in vivo* but also validate the effect M2 on the inhibition of apoptosis *in vivo* for the first time through an innovative approach. Without doubt this work contributed to better understanding of M2 role in the physiopathology of infection as well the immune response at the cellular level. In the future, it would be interesting to validate our results in a mouse with controlled gene expression system where the gene of interest is expressed temporally as well as spatially controlled manner.

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